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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2014

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Stolzenburg, S. (2014). *Epigenetic editing using programmable zinc ginger proteins: inherited silencing of endogenous gene expression by targeted DNA methylation*. [Thesis fully internal (DIV), University of Groningen]. [S.n.].

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Epigenetic Editing using programmable Zinc Finger Proteins

Inherited silencing of endogenous gene expression
by targeted DNA methylation

Sabine Stolzenburg

The research described in this thesis was financially supported by a Rosalind Franklin fellowship, UMCG, NCI/NIH grants and DoD awards.

Printing of this thesis was financially supported by:

Synvolux Therapeutics B.V.

The University of Groningen

Cover design: Sabine Stolzenburg
Layout: Sabine Stolzenburg and Off Page printing
Printed by: Off Page printing

ISBN: 978-90-367-7053-8 (printed version)
ISBN: 978-90-367-7052-1 (electronic version)

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Inherited silencing of endogenous gene expression by targeted DNA methylation

PhD thesis

to obtain the degree of PhD at the
University of Groningen
on the authority of the
Rector Magnificus Prof. E. Sterken
and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on

Wednesday 4 June 2014 at 16.15 hours

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Chapter 1

General Introduction

EPIGENETICS

The term epigenetics was coined by Waddington who defined epigenetics as 'the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being' (1). This definition has evolved over time to the current understanding of epigenetics referring to 'the study of heritable changes in gene expression that occur independent of changes in the primary DNA sequence' (2). The basic unit of chromatin comprises the nucleosome which consists of approximately 146 base pairs (bps) of DNA wrapped around an octamer containing two copies of each of the core histones H2A, H2B, H3 and H4. Biochemical modifications on DNA and histones, as well as the nuclear context, influence the three dimensional structure of chromatin. The main covalent chemical modification on DNA itself is the methylation of cytosines followed by a guanine base (CpGs). Additionally, post-translational histone modification (PTMs), nucleosome positioning and the expression of non-protein coding RNAs (ncRNAs) are important epigenetic modifications. Specific combinations of epigenetic modifications are thought to orchestrate chromatin density and promoter accessibility, and to generate an "epigenetic code" that ultimately regulates stable gene expression patterns. Aberrations in the epigenetic code (epi-mutations) play a major role in disease development and progression, especially in cancer, as well as in autoimmune diseases and neurological disorders (3-5). The ability to modulate the epigenome at will at specific target sites associated with disease would have a huge impact towards novel therapeutic approaches and on our current understanding how the epigenome is controlled. In this thesis, we reached out to modify the expression of specific endogenous target genes by induction of artificial DNA methylation.

DNA methylation

Two active classes of DNA methyltransferase (DNMT) enzymes regulate DNA methylation. The classic model defines the function of DNMT1 to preserve DNA methylation after replication, while DNMT3A and DNMT3B exhibit mostly *de novo* methylation activity; however they also appear to contribute to the maintenance of DNA methylation (6,7). A third member of the DNMT3 family (DNMT3L = DNMT3A-like) is catalytically inactive but stimulates the activity of DNMT3A and 3B (8).

The methylation of cytosines (5mC) in the context of CpG dinucleotides has been studied extensively (9). In promoter regions CpG dinucleotides often cluster in so-called CpG islands (CGI) and more than half of the human gene promoters contain CGI (10,11). These CpG-rich promoters are usually unmethylated, with a few exceptions of tissue-specific methylation during development (12,13). To a lesser extent, CpG dinucleotides are also found in gene bodies and in CpG island shores, which are regions directly adjacent to CGI.

Currently, the role of CpG methylation in the direct causality of gene silencing is unclear and its effect on gene expression has been found to be highly dependent on the location within the gene (9,14). However, DNA methylation of CpG shores, CGIs and exon 1 is directly associated with inactive gene expression (15,16). Furthermore, DNA methylation is involved

in the permanent silencing of the inactive X-chromosome, suppression of transposable elements and genomic imprinting (17-20). In contrast, CpG methylation within gene bodies is mostly correlated with active gene expression however can also function to prevent anti-sense transcription or gene expression from alternative promoters (19,21). Hence proximal to the transcription start site CpG methylation blocks the initiation of transcription, but does not prevent elongation of transcription when present at gene bodies (9). Recent findings, that exons are more heavily methylated than introns and that the methylation intensity changes at exon-intron boundaries has led to the suggestion, that gene-body methylation has also a role in alternative splicing (22-25).

Histone modifications

Besides DNA methylation, post-translational modifications (PTMs) on histone tails protruding from the four core histones (H2A, H2B, H3 and H4) influence the chromatin structure. The balance of active and repressive chromatin modifications maintains genome-wide expression states (26). Histone tails undergo a number of PTMs, including acetylation, methylation, phosphorylation, ubiquitinylation and ADP ribosylation, orchestrating gene expression in a combinatorial fashion (27). The best described PTMs are methylation and acetylation of lysine (K) residues located at the N-terminal tails that protrude from the core histones H3 and H4. Lysine methylation occurs in form of mono-, di- and tri-methyaltion according to the number of methyl groups bound.

While histone acetylation is associated with active gene expression, lysine methylation is highly context dependent. The trimethylation of H3K4, K36, and K79, and the monomethylation of H4K20, are associated with gene activation; whereas, the di- and trimethylation of H3K9 and H3K27 are associated with gene repression. Exceptions are developmental genes in embryonic stem cells. They are marked by an epigenetic "bivalent state" harbouring both transcriptionally active H3K4me3 and transcriptionally repressive H3K27me3 methylation marks (28). The simultaneous presence of active and repressive marks keeps these genes poised for either expression or repression.

The enzymes orchestrating PTMs can be divided into 'writers' and 'erasers' (29). Additionally 'readers" exists which recognise a histone mark, for example, chromo- and bromo- domains read histone acetylation and histone lysine methylation, respectively. The writers, such as histone acetyltransferase (HATs) and histone methyltransferases (HMTs) place an epigenetic mark (30,31). The erasers, for example histone deacteylases (HDACs and sirtuins) and histone demethylases (HDMs), remove these marks from the histones. There is a tight interconnection between the PTMs on histone tails, whereby the PTMs influence each other, and also link PTMs and DNA methylation. For instance, it is now well established that DNA methylation and methylation at H3K4 are mutually exclusive, suggesting that H3K4 methylation may protect DNA from *de novo* methylation (32-35).

For the maintenance of DNA methylation during replication, the enzyme ubiquitin-like PHD and RING-finger containing 1 (UHRF1) has been identified as being essential in targeting DNMT1 to the replication fork (36,37). UHRF1 contains the SET and RING-associated domain (SRA) that recognises hemimethylated DNA and a tandem tudor domain

(TTD) which binds to H3K9me3 (38,39). Subsequently UHRF1 recruits DNMT1 which serves as a platform for other histone modifiers, such as HDACs and G9a. These findings show a close interaction between DNA methylation and histone modification, also referred to as epigenetic "cross-talk" (40).

Non-coding RNAs

MicroRNAs (miRNAs) are small non-coding RNAs, consisting of approximately 22 nucleotides, that regulate the expression of hundreds of genes by targeting their mRNA post-transcriptionally (41). By binding to the partially complementary target site in the 3'-untranslated regions (3'-UTR) of mRNAs, miRNAs regulate gene expression by inducing direct mRNA degradation or translational inhibition (41). To date, the expression profiles of a large number of miRNAs have been found to be dysregulated in cancer, and indeed, many miRNAs act as tumour-suppressors or oncogenes (42). MicroRNAs can regulate and be regulated by epigenetic mechanisms: for example, promoter silencing through DNA methylation of miRNA 124a has been associated with cancer and down-regulation of miRNA-148, miRNA-34b/c and miRNA-9 lead to metastasis (43-45).

Epigenetic modifications in cancer

In cancer cells the epigenetic landscape is extremely disrupted. Hallmarks of cancer cells are global DNA hypomethylation, resulting in genome instability and local hypermethylation which can cause specific promoters to be silenced (46). Genes affected by a gain of DNA methylation include tumour suppressor genes (p53, *BRCA1*, *MASPIN*) and genes involved in important cellular pathways (cell cycle control, apoptosis and DNA repair) (5,47-49). Loss of DNA methylation at oncogenic promoters causes the re-expression of the affected oncogene. Other well described epigenetic alterations in cancer cells are modifications on histones, nucleosome positioning and expression of non-coding RNAs (2). The global loss of H4K16ac, H4K20me3 and H3K4me3 as well as gains of H3K9me and H3K27me3 also play major roles in cancer (5).

With growing knowledge of the epigenome of cancerous versus normal tissue, new targets for cancer drug therapy have been found. To date, the US Food and Drug Administration (FDA) has approved four epigenetic drugs, two DNMT inhibitors and two HDAC inhibitors. Both DNMT inhibitors are approved for the treatment of high risk myelodysplastic syndrome (MDS), a precursor to leukaemia (50,51). The DNMT inhibitors 5-azacytidin and 5-aza-2-deoxycytidine are nucleoside analogues that incorporate into DNA and RNA, or DNA only, and thereby inhibit all three active DNA methyltransferases. The two approved HDAC inhibitors are used for the treatment of cutaneous T-cell lymphoma (CTCL), and two additional HDAC inhibitors are currently being tested in clinical trial III studies for the treatment of lymphomas and non-small lung cancer (52). Although already in clinical practice, epigenetic drugs are not gene-specific and affect genome-wide DNA methylation and histone acetylation in a transient manner. With this in mind, there is an urgent need for therapeutic approaches with higher specificity.

Targeted gene regulation

Conventionally, specific up- and down-regulation of gene expression is achieved by cDNA and siRNA transfection, respectively. Although both approaches have been successful in the past, one major limitation is their instability over time, due to short half-life times and because newly synthesized mRNA molecules need to be constantly targeted. The administration of cDNA requires the harmful integration in the genome for permanent effects and furthermore, will lead to the expression of one isoform of the protein only instead of expressing all isoforms of a protein in their natural ratios.

The locus-specific regulation of gene expression at the DNA level is, therefore, a promising area to explore in the effort to correct aberrant gene expression. Targeted gene specific up- and down-regulation of gene expression can be achieved by artificial transcription factors (ATFs). ATFs consist of, at a minimum, a sequence specific DNA-binding domain (DBD) coupled to an effector domain. The advantages of the ATF approach include 1) only two copies of DNA have to be targeted per cell instead of silencing many mRNA transcripts using siRNA; 2) in contrast to the introduction of cDNA, all splice variants of a gene will be induced; and 3) mitotically stable gene modulation is possible dependent on the linked effector domain (*see Study Aims*).

While the DBD mediates sequence specific recognition of the target region, the effector domain mediates, directly or indirectly, activation or repression of gene expression. The most successfully used transcriptional activator is the herpes simplex viral protein 16 (and its tetrameric repeat VP64) domain, and the most commonly fused repressor is the Krueppel-associated Box (KRAB, or super KRAB domain (SKD)). Both of these effector domains recruit cellular expression modulators to form a complex and regulate gene expression transiently (53). In order to regulate endogenous gene expression in a permanent manner, epigenetic modifying enzymes, or their catalytic domains, can be used, introducing directed epigenetic marks and promoting inherited modulation of the chromatin structure which consequently alters target gene expression [(54) reviewed in (55) and see below]. Recently, several groups have published results on targeted epigenetic silencing using DNA methyltransferases and histone modifying enzymes (8,56-58).

To target gene expression, different sequence recognition modules have been developed, including triple-helix forming oligonucleotides (TFOs) (Figure 1A); synthetic polyamides; designer zinc finger proteins in ATFs (Figure 1B); and, more recently, transcription activator like effectors (TALEs) (Figure 1C) and the Cas9 RNA-guided DNA binding proteins of the clustered regularly interspaced palindromic repeat (CRISPR) system (Figure 1D) (59-61).

DNA binding domains (DBD)

Triple-helix forming oligonucleotides (TFOs) and Polyamides

TFOs are single polynucleotide strands that bind through consecutive hydrogen bonds to their target sequence in the major groove of double stranded DNA, forming a triple helical structure (Figure 1A). TFOs only bind purine-rich regions efficiently and have low

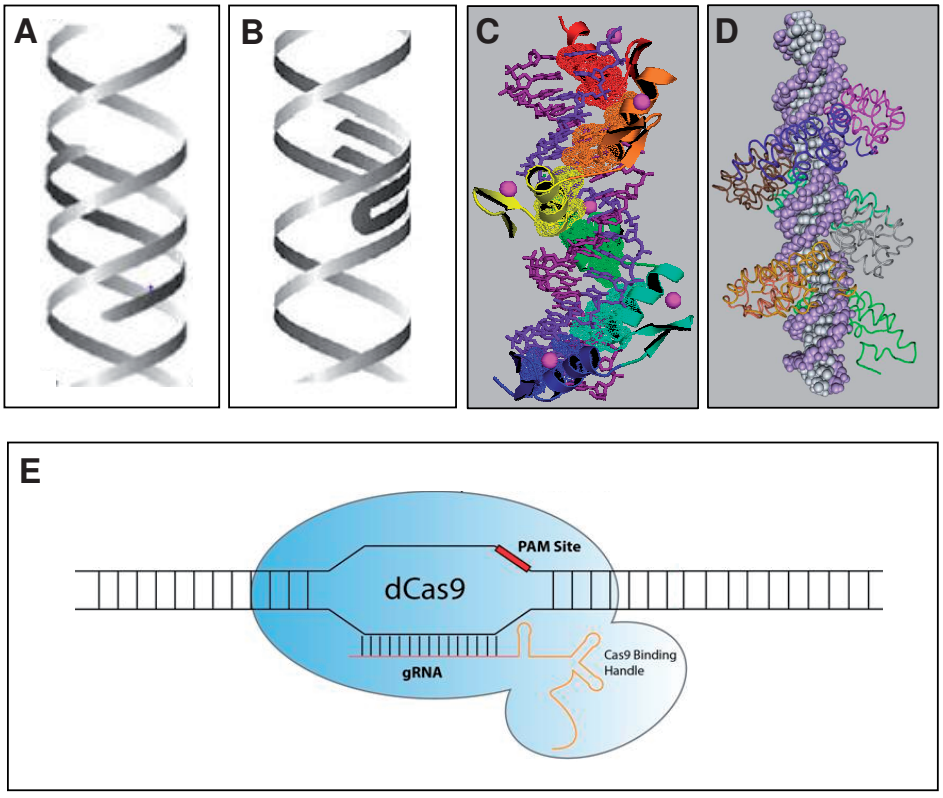

Figure 1. DNA binding domains (DBD) used to target endogenous gene expression. A) Schematic illustration of a triple helix forming oligonucleotide binding the major groove of double stranded DNA (adapted from Uil et al 2003). B) schematic illustration of a hairpin polyamid binding the monor groove of double stranded DNA (adapted from Visser et al 2006). C) Crystal structure of a 6-finger ZFP (each pink circle symbolizes a zinc atom of one ZF domain) bound to the major grove of the DNA double-helix (in magenta and purple) D) Crystal structure of a transcription activator like effector (TALE) bound to the double-helix (grey and purple). C) Schematic illustration of a clustered regularly interspaced palindromic repeat (CRISPR) CRISPER-associated (Cas) protein (light blue) and the guide RNA (gRNA) bound to the DNA.

stability under physiological conditions (62). Nonetheless, fused to epigenetic effector domains, TFOs have been used to direct DNA methylation to plasmid DNA (63). Synthetic polyamides can also be used as DNA binding domains in order to modulate gene expression, whereby two anti-parallel polyamide stretches (Figure 1B), consisting of hydroxypyrrole (Hp) imidazol (Im) and pyrrol (Py), can built a hairpin formation through side-by-side amino acid pairing (64,65). These hairpin structures bind to specific base pairs in the minor groove of double helical DNA by Hoogsteen hydrogen bonds. The drawback, however, of synthetic polyamides is their relatively short recognition site in the DNA, which cannot be extended simply by elongation of the polyamide stretches.

Transcription activator-like effectors (TALEs)

A newly described class of designer DNA binding proteins are transcription activator-like effectors (TALEs), which have been derived from plant pathogenic bacteria to modulate host gene expression (Figure 1D) (65). They are injected into the plant cells and imported into the nucleus, where they bind effector-specific sequences of the host cell genome and activate transcription. A central domain determines the DNA-binding specificity of TALEs. Typically, this domain consists of 15.5 to 19.5 repeats (though variants from 1.5 to 33.5 repeats have been recorded); with each repeat encoding 33 or 34 amino acids. This array of repeats is followed by one truncated repeat with 20 amino acids (60). Each monomer differs at amino acid positions 12 and 13, a region called repeat variable di-residue (RVD). These hypervariable residues mediate binding to the DNA target site; whereby, each RVD recognises one nucleotide within the DNA-binding site (HD=C, NI=A, NG=T, NN=G). Subsequently, transcriptional activators, repressors or nucleases can be fused to the TALE DBD for targeted gene expression modification. Targeting efficiencies of the TALE DBD range from 25 to 95 percent (66,67), and new assembly methods are now available to improve the generation of more efficient TALEs (68,69). Considerable progress has been made in the design, development and characterisation of TALEs in the last two years (70). There are, however, two major disadvantages of TALEs: 1) their high number of tandem repeats, making them prone to rearrangements upon expression in the target cells (71); and 2) their large protein size, which may cause problems in delivery, and may hamper their ability to access mammalian heterochromatin (72). As mentioned previously, the field of TALEs research is still in an early stage and more work is needed to optimize and verify the target specificity of TALEs *in vivo* (67).

Clustered regulatory interspaced palindromic repeats (CRISPR) /CRISPR-associated (Cas)

The clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated (Cas) targeting systems is also derived from bacteria- (and archaea). CRISPR/Cas systems have evolved as adaptive immune defence mechanisms that rely on short RNAs for sequence-specific detection and degradation of foreign nucleic acids (73,74). When foreign DNA enters the host cell, parts of the invading DNA are trapped as 'spacers' between 'repeats'. Transcription of the repeat-spacer fragment results in a precursor CRISPR RNA (pre-crRNA), which is processed into crRNA carrying one spacer and one repeat (74). In the type II CRISPR system, a trans-activating crRNA (tracrRNA), complementary to the repeat sequence of crRNA, builds a complex with the Cas9 protein and this complex catalyses destruction of the invading nucleic acid (73). The system is artificially modified for purposes of efficiency in a way that the tracrRNA and crRNA are expressed as fusions, termed single guide RNA (sgRNA), where the spacer fragment is exchanged by the desired target sequence (75). The Cas protein is then fused with a nuclear localisation signal and co-delivered with the sgRNA. This system can be further modified: whereby, the Cas9 protein is converted into an inactive form and fused to transcriptional activators and repressors, respectively (57) (Figure 1E).

Zinc finger (ZF) proteins

The best studied approach for specific DNA binding is the use of designer zinc finger (ZF) proteins (76). ZF proteins are naturally occurring transcription factors forming the largest group of all transcription factors in the human genome (77). They consist of approximately 30 amino acids: wherein, a stretch of seven amino acids is responsible for the recognition of three to four base pairs (bps) in the major groove of double stranded DNA (Figure 1C). During ZF-binding, the amino acids at position -1, 3 and 6 in the alpha-helix of the ZF protein recognise the third, second and first nucleotides of the target sequence in the 5'-3' DNA strand. In 1996, Kim and Berg published the crystal structure of a designed ZF protein, which revealed an additional bond between certain amino acid at position two in the ZF alpha helix and the fourth base in the antisense strand, which is at the same time the complement nucleotide of the second triplicate, recognised by the amino acid 6 of the second ZF protein (78). Klug and colleagues in 1994 engineered the first ZF protein successfully targeting the BCR-ABL fusion oncogene (79). Since this pioneering work was conducted, engineered ZF proteins have been used to target a multitude of endogenous genes (Table 1) and their relatively small size and low immunogenicity are major advantages when compared to other DNA targeting proteins. Importantly, the potential of ZF proteins for therapeutic applications is currently explored in clinical trials (80). As explained below, three major strategies are now available to generate ZF proteins: 1) engineering of designed ZF proteins by modular design; 2) the OPEN strategy (Oligomerized Pool Engineering); and 3) the selection of ZF proteins from degenerated ZF libraries.

Generation of Zinc Finger (ZF)-based DNA-binding domains

Modular design of artificial transcription factors (ATFs)

The engineering of polydactyl ZF-based ATFs is based on the modular character of single ZF proteins. By assembling multiple building blocks of single ZF proteins (each recognising 3 bps), a DNA stretch of a multiplicity of three nucleotides can be targeted. The first step in engineering a custom made ZF protein is the determination of a suitable target region in the promoter sequence of the gene of interest. (*Note:* This step is performed *in silico*, and does not take the endogenous chromatin structure into account.) The second step is the selection of building modules targeting 5'-GNN-3' 5'-ANN-3', 5'-CNN-3' and 5'-TNN-3' triplicates coding for the seven amino acids that complex with the DNA target sequence (81,82). By stitching together six ZF modules a specific region of 18 bps can be recognized, which theoretically occurs only once in the human genome. Assembly of the single ZF-modules using the canonical peptide linker TGEKP generates the DBD of the ATF. The DBD is then tested for its ability to bind its target sequence (83). Although the modular design does not take the chromatin structure of the target region into account (which is important for ZF-binding to its endogenous target region), in a study by Segal *et al* 70 of the 80 modular designed ZF proteins showed specificity for their target region (83). In order to achieve successful ZF-binding, it is recommended that multiple sets of ZF DBD be engineered and that the chromatin accessibility of the target region be taken into account (84). Alternatively

since 2008 ZF proteins can be purchased from Sigma-Aldrich (85). The third and final step in the generation of an ATF is the selection and fusion of an effector domain. Several effector domains are now available, distinguishable by their direct or indirect modulation of gene expression. Depending on whether the gene of interest is aimed to be up- or down-regulated, different effector domains can be chosen (Table 1 and 2).

Table 1. Endogenous targets regulated by ZF protein transcription factors

Endogenous gene	Regulation	bp target site	Effector Domain	Reference
Bax	↑	15	VP16	(88)
BCR-ABL	↓	9		(79)
CHK2	↓	18	SKD	(89)
CCK2R	↑	9	VP16	(90)
ELN	↑	9	VP16	(91)
EpCAM	↑	18	VP64/SKD	(92) *this thesis*
EPO-1	↑	9	VP64	(93)
ErbB2/HER-2	↑↓	18	VP64/SKD	(81,94-97)
ErbB-3	↑↓	18	VP64/SKD	(81,96)
β-Globin	↓	18		(98)
GDNF	↑	18	P65	(99)
γ-Globin	↑	18	VP64	(100-102)
Huntingtin	↓	12-54	SKD	(103)
HIV	↓	9	SKD	(104-106)
IGF2/H19	↑↓	9	VP16/p65/v-ErbA	(107)
MASPIN	↑	18	VP64	(47,108)
MDR1	↑↓	15	VP16/SKD	(109)
OCT-4	↑↓	18	VP16/SKD	(110,111)
PEDF	↑	18	p65	(112)
PLN	↓	18	SKD	(84)
PTHR1	↑	9	VP16/p65	(113)
PPARγ	↓	18	SKD	(85)
SOX2	↓	18	SKD	(114) *this thesis*
hRHO	↑↓	18	VP64/SKD	(115)
hTERT	↓	12	SKD	(116)
Utrophin	↑	12/9	VP16	(117)
VEGF-A	↑↓	9/19	VP16/p65/SKD FDTL/SID/SRDX	(101,118-122)

Listed are endogenous genes being positively and negatively regulated by ZF ATFs using transient effector domains. Illustrated is whether the gene was up- (↑) or down- (↓) regulated with regards to the size of the endogenous target sequence and the effector domains used. VP-16 = herpes simplex virus protein VP-16, VP64 = tetrameric repeat of VP-16, SKD = Kruppel-associated Box domain of KOX1, p65 = subunit of the human NF-κB transcription factor, SID = mSin3 interaction domain of the transcriptional repressor MAD, FDTDL = transactivation domain of β-catenin, SDRX = repressor domain of Arabidopsis thaliana SUPERMAN.

Table 2. Endogenous targets modulated by Epigenetic Editing

Target gene	DBD	bp target site	Effector Domain	Reference
Grm2	TALE	14	24 modifiers	(57)
HBB	TALE/ZF protein	20/ 18-20	TET1	(144)
Her2/Neu	ZF protein	18	G9a/ SUV39-H1	(56)
ICAM	ZF protein	18	TET2	(145)
KLF4	TALE/ZF protein	17, 18/ 18, 19	TET1	(144)
MASPIN	ZF protein	18	DNMT3A	(136)
Neurog2	TALE	16	32 modifiers	(146)
Nos2	ZF protein		TDG	(147)
SCL	TALE	18	LSD1	(58)
SOX2	ZF protein	18	DNMT3A	(136) *this thesis*
RHOXF2	TALE	20	TET1	(144)
VEGF-A	ZF protein	9/19	DNMT3A-DNMT3L and G9a/ SUV39-H1	(56,138,148)

Listed are endogenous target genes subjected to Epigenetic Editing. Indicated is the DNA binding domain (DBD) used for targeting: Zinc finger (ZF) protein and transcription activator like effector (TALE), respectively, the size of the endogenous target sequence and the epigenetic modifiers used: TET1/2: ten-eleven translocation 1/2 = catalyzes DNA demethylation, TDG: Thymidine DNA glycosylase = catalyzes DNA demethylation. LSD1: Lysine-specific demethylase 1 = catalyzes H3K4 demethylation, DNMT3A = catalyzes *de novo* DNA methylation, G9a/SUV39-H1 = catalyzes H3K9 methylation.

Oligomerized Pool Engineering (OPEN)

The OPEN selection system was developed by the Zinc Finger Consortium and first described by Maeder *et al* (86). This method requires pools of pre-selected ZFs, constructed by cassette mutagenesis and obtained from randomised ZF-libraries. In the first of two steps of OPEN selection, ZF-libraries, consisting of three pools of ZF proteins are introduced into bacterial two-hybrid (B2H) selection strain cells harbouring the target site of interest driving expression of a resistance gene. Cells are plated on selection media, surviving colonies are isolated and ZF encoding DNA is salvaged. In the second selection step, ZF encoding phagemids are re-introduced into a new strain of B2H cells and plated on gradient plates, then colonies are picked and ZF protein encoding DNA is isolated by plasmid purification. Both ZF engineering strategies mentioned lack the ability to take the endogenous chromatin structure into account. This is significant because the efficiency of the binding in the endogenous target region may be compromised as the chromatin environment at the targeting locus is neglected.

Selection of ZF proteins from a ZF-Library

Importantly, the modular character of ZF proteins allowed their combinatorial assembly for the generation of ZF-libraries. These libraries can consist of either 3-finger or 6-finger ZF proteins, targeting GNN or ANN triplicates, respectively (87). Application of the ZF-library enables cell culture based selection of ZF proteins binding to endogenous targets. To this

end, a pool of ZF proteins linked to effector domains are retrovirally delivered into host cells, followed by a selection step specifically collecting cells, which acquired a desired phenotype, eg drug-resistance or surface-antigen expression (Figure 2). From the selected cells, ZF proteins are recovered by PCR and subcloned into the original expression vector for additional rounds of selection. After multiple rounds of selection, ZF proteins can be isolated specific for an endogenous target gene. The rational for this experimental design is that the library consists of combinatorial assembled ZF proteins and for each possible genomic target sequence at least one targeting ZF is represented in the library pool. In theory, this enables the recognition of any possible target region. Experimentally, however, the library screen only modulated two out of ten genes (87).

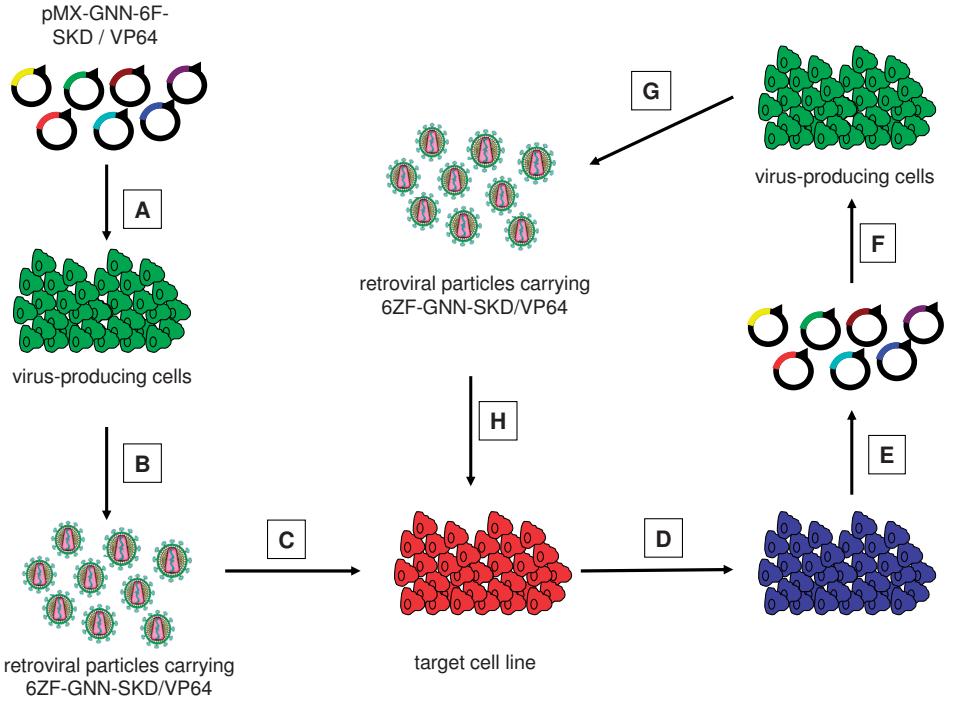

Figure 2. Genome-wide screening using a ZF-library. A) Plasmid DNA pools coding for $2x10^7$ ZF proteins linked either to the repressor SKD or the activator VP64 is introduced into virus producing Gag-Pol cells. B) Virus-producing cells secrete viral particles coding for $2x10^7$ individual ZF proteins. C) Virus particles are used to infect a first target cell line. D) Cells with a desired phenotype are selected (eg drug-resistance or Flourescence Activated Cell Sorting, based on cell-surface marker expression) and subcultured. E) Isolation of ZF proteins which induces the acquired phenotype of interest. F) Rescued ZF protein DNA is used to transfect virus-producing Gag-Pol cells for a second round of selection. G) = B) and H) = C)

Effector domains for transcriptional up-regulation

For specific up-regulation of gene expression, activating effector domains, like the herpes simplex virus protein VP16 and its tetramer VP64, consisting of four VP16 activation subunits, can be fused to the ZF protein (47). This will lead to a transient up-regulation of the target gene (108). It has been suggested that VP16 activates gene expression by facilitating the assembly of an RNA polymerase II pre-initiation complex and by interacting with chromatin-remodelling enzymes, such as, components of the SWI/SNF complex (123). Liu *et al* fused the NF-κB transcription factor p65 domain to a *VEGF-A* targeting ZF protein and, as a result, induced multiple splice variants of VEGF-A mRNA (101). Both activators mediate transcription through the recruitment of TATA-box-binding protein (TBP) and TFIIB (124,125).

Effector domains for transcriptional down-regulation

The most commonly used repressive effector domain is the human Krüppel-associated box (SKD) domain of the KOX1 protein. SKD consists of an A and B box wherein a domain of 45 amino acids in the A box is defined to function as a transient repressor of transcription (126). It has been suggested that SKD acts via three different pathways: first by recruiting enzymes responsible for changing the epigenetic environment; second by interfering with the TATA box complex assembly; and third by relocating the gene to heterochromatin compartments in the nucleus. Recently, Juarez-Moreno et al found that SKD also functions as an activator of gene expression (111). Other powerful tools with a transcriptional repressive effect are the mSin3 interaction domain (SID), and the ligand dependent thyroid hormone receptor (TR) or its viral relative vErbA (127).

The above described activation or silencing mechanisms are based on indirect recruitment of co-activators and co-repressors and, therefore, are less likely to result in stable modifications. After dissociation of the ATF from its endogenous binding site, the previous expression state of the cell will be restored. A more direct and permanent approach to truly modulate gene expression in a permanent manner can be achieved by the direct targeting of epigenetic enzymes or catalytic domains thereof to specific target sites [this thesis and reviewed in (55)].

Epigenetic modulators of gene expression

DNA methylation and PMTs are stably transmitted over cell generations, and DNA methylation on gene promoters and the first exon is typically associated with transcriptional repression (9,15); therefore, it seems indicative to induce targeted DNA methylation in aberrantly activated promoters to permanently silence their expression. Various DNMTs (of bacterial, mouse and human origin) have been explored to target oligonucleotides, plasmid DNA in bacteria or as reporter transfected into mammalian cells, integrated promoter-sites, mitochondrial and viral DNA (55,128-135). It has been 15 years, however, since the first targeting study by Xu *et al*. using a ZF-methyltransferase, until an endogenous promoter in the human genome has been successfully down-regulated through targeted

DNA methylation by an engineered ZF protein fused to the human DNMT *in vitro* [this thesis (136,137)] and *in vivo* (*this thesis*).

Many other epigenetic enzymes serve as potential candidates for targeting studies when fused to engineered DBD [reviewed in (55)]. Pioneering work in epigenetic reprogramming of an endogenous target was conducted by Snowden et al by fusing the histone methyltransferase G9a to a VEGF-targeting ZF protein and inducing H3K9 di-methylation, and consequently down-regulating gene expression (138). Recently, several more publications have reported successful epigenetic targeting using different DBDs (table 2).

EpCAM and SOX2

In this study, the modulation of endogenous gene expression using ATFs is addressed. As model genes for targeted regulation of endogenous promoters the Epithlial Cell Adhesion Molecule (*EpCAM*) and the transcription factor Sex determining Region Y Box 2 (*SOX2*) were explored. Both genes are important players in the development and progression of cancer as described below.

Epithlial Cell Adheasion Molecule (EpCAM)

EpCAM has been identified as a highly immunogenic tumour antigen. It has been described by many independent research groups, hence there are a myriad of different synonyms used for the antigen dependent on the monoclonal antibody used to recognise it (139). *EpCAM* was first described in 1979 when it was found as an antigen on colon carcinomas (140). *EpCAM* is a cell-cell adhesion molecule of 30kDa consisting of an extracellular domain (EpEX), a transmembrane and an intracellular domain (EpICD). It is well known that *EpCAM* plays a pivotal role in cell signalling, proliferation, migration, embryogenesis and maintenance of stem cell growth (141-143).

A close functional link has been reported between *EpCAM* and the activation of the Wnt-/beta-catenin signalling pathway: after cleavage of *EpCAM*, intracellular EpICD builds a complex with the FLH-2, beta-catenin and the transcription factor Lef-1, which induces expression of c-myc and cyclin A and E (142). Ralhan *et al* reported that a nuclear and cytoplasmic EpICD accumulation is found in human epithelial cancers (149). *EpCAM* has been found over expressed in a multitude of carcinomas, especially playing a role in tumour initiation of colon, breast, liver and pancreatic cancers (143,150-156). Furthermore, siRNA knock-out of *EpCAM* expression has been found to reduce the oncogenic phenotype of breast cancer cells (157).

Although *EpCAM* is over expressed in many human carcinomas of epithelial origin, and has been subject in a number of antibody and vaccine-based clinical trials, it cannot be strictly considered as an oncogene: In some cancer types, *EpCAM* over expression is reported to correlate with a positive outcome and it seems to act as a tumour suppressor gene [reviewed in (139)]. It is assumed that *EpCAM,* according to its environment, behaves either like an oncogene or a tumour suppressor gene: for example, metastases often have a down-regulated *EpCAM* expression compared to the primary tumour (158). The ability

to specifically up- or down-regulate *EpCAM* expression at the endogenous level could elucidate its role in normal and malignant cells. Epigenetic gene expression regulation has been demonstrated by us and DNA binding domains have also been engineered (63,159-161). This and the fact that *EpCAM* function in cancer remains very unclear, and even contradictory, makes *EpCAM* a good target gene for both up- and down-regulation, via targeted expression modulation using ATFs in cancer gene therapy.

SRY (Sex determining Region Y) Box 2 (SOX2)

SOX2 is a member of the SRY-related High Mobility Group (HMG) box transcription factors and is involved in the regulation of embryonic development, determination of cell fate, and the maintenance of self-renewal in embryonic and adult stem cells. During differentiation, *SOX2* undergoes epigenetic silencing and most adult tissue, including breast, are *SOX2*-negative (162,163). The epigenetic mechanisms involved in *SOX2* silencing are promoter DNA methylation and methylation at two distinct regulatory regions, ~4kb up- and down-stream of the transcription start site (164). Furthermore, *SOX2* is regulated through the interaction of miRNA-126 (165). In recent years a growing number of studies showed over-expression of *SOX2* in a multitude of malignancies, giving strong evidence of the oncogenic potential of *SOX2* (166-169). In glioblastoma, prostate, lung, liver and breast cancers, *SOX2* has been identified in subpopulations of cancer cells with cancer initiating properties (167,168,170-173). These populations of poorly differentiated cells exhibit stem cell-like characteristics, such as sustained proliferation and drug resistance (154,174-177). Down-regulation using *SOX2* targeting siRNA in breast and lung cancers, and osteosarcoma, leads to a less tumorigenic phenotype *in vivo* (169,178-180). Permanent down-regulation of *SOX2* can have many applications in fundamental research, reprogramming and differentiation studies, and maybe even in the treatment of different malignancies.

AIMS AND OUTLINE OF THIS THESIS

The aim of this thesis is to induce the stable silencing of an endogenous target gene, by directed DNA methylation and inducing inherited transcriptional memory. Several crucial variables had to be addressed to achieve this aim: 1) the endogenous locus is targeted; 2) the target gene is artificially regulated; 3) directed DNA methylation leads to transcriptional silencing 4) the epigenetic mark and transcriptional silencing is transmitted over cell generations.

To modify endogenous gene expression, ZF proteins binding their target site in the chromatin context of the nucleus need to be identified. Different methods have been previously described, including the modular design and the *in vivo* selection from a ZF-library (**Chapter 2** and **Chapter 3**). Our first aim was to target the endogenous promoter of EpCAM using previously, by modular design, generated ZF proteins (159). Regulatory efficiency of these ZF proteins on *EpCAM* expression at reporter level had been shown before (159). As the transient transfected ZF proteins did not modify endogenous gene expression (**Chapter 3**), we intended to use epigenetic drugs to open the endogenous

chromatin structure to facilitate a more relaxed chromatin environment which could allow the modular designed ZF proteins to access their target site (**Chapter 3**). Additionally, we set out to identify new ZF proteins to bind the endogenous *EpCAM* promoter by screening a ZF-library which allows the selection of potent ZF proteins based on their ability to regulate endogenous gene expression (**Chapter 3**).

Alternatively to the transient transfection, ZF proteins can be delivered by viral transduction, nanoparticles and direct protein delivery (47,181,182). Therefore, the previously modular assembled ZF proteins were delivered by retroviral transduction. This led to a strong regulation of endogenous *EpCAM* expression, as published in a separate manuscript by Gun *et al* (92).

As an additional target gene, we chose *SOX2* to address whether ZF fusion proteins are a powerful tool to revert an oncogenic phenotype in breast cancer. *SOX2* is a master regulator for pluripotency and appears to be involved in the initiation of cancer and is especially over expressed in the basal-like subtype of triple negative breast carcinomas (173). Endogenous down-regulation of *SOX2* using RNAi has previously shown to decrease the tumorigenic phenotype in lung, breast and ovarian cancers (169,183,184), however, long-term studies in animals have shown that the effect of RNAi is only transient. Therefore, we aimed to target *SOX2* at the gene expression level and generated ZF proteins targeting the endogenous *SOX2* promoter (**Chapter 4**).

In **Chapter 4** we intended to validate the choice of the target gene and that ZF proteins are a powerful tool to control the expression using the transient effector Krueppel-associated box domain (SKD). Fused to *SOX2* targeted ZF proteins we aimed to decrease the oncogenic potential of breast cancer cells in a xenograft mouse model. Silencing of *SOX2* did indeed result in a decrease of the tumorigenic phenotype of the transplanted cells, inhibition of tumour growth and overall change in the morphology of the tumour to a phenotype comparable to normal breast (**Chapter 4** and **Chapter 6**).

As the effect of SKD-mediated down-regulation is also only transient, we intended to induce stable silencing of endogenous gene expression by targeted cytosine methylation. As a proof of concept we aimed to actively methylate SOX2 and MASPIN by using targeted ZF proteins fused to the human DNA methyltransferase DNMT3A (**Chapter 5**). Endogenous DNA methylation has been reported to be faithfully transmitted to daughter cells over cell generations (185), therefore, in **Chapter 5** and **Chapter 6** we explore whether artificial DNA methylation leads to gene silencing and is maintained during mitosis. Furthermore, we set out to confirm previous findings that propagation of DNA methylation involves the presence of UHRF1 during replication (36).

As artificially induced DNA methylation was transmitted over cell generations in cell culture experiments we set out to evaluate the *in vivo* longevity of the incorporated DNA methylation in a xenograft mouse model (**Chapter 6**).

In **Chapter 7** the most important findings of this thesis are summarised followed by a discussion of these findings in the context of the existing literature and an exploration of the potential for future research that stems on from these findings (**Chapter 8**).

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Chapter 2

Modulation of gene expression using Zinc Finger based Artificial Transcription Factors

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Methods Mol Biol. 2010;649:117-32.

SUMMARY

Artificial transcription factors (ATFs) consist of a transcriptional effector domain fused to a DNA binding domain such as an engineered zinc finger protein (ZFP). Depending on the effector domain, ATFs can up- or downregulate gene expression and thus represent powerful tools in biomedical research and allow novel approaches in clinical practice. Here, we describe the construction of ATFs directed against the promoter of the Epithelial Cell Adhesion Molecule and against the promoter of the RNA component of telomerase. Methods to assess DNA binding of the engineered ZFP as well as to determine and improve the cellular effect of ATFs on (endogenous) promoter activity are described.

1. INTRODUCTION

An increasing number of diseases are described to be associated with aberrant gene expression profiles due to genetic mutations or epigenetic factors. Turning gene expression on or off at will in a gene-specific way thus represents a powerful tool in biomedical research and opens up new possibilities to regulate the activity of (as yet) undruggable targets in clinical practice. Several approaches are currently being explored to design such gene-specific Artificial Transcription Factors (ATFs) that, at a minimum, consist of a sequence specific DNA recognition module coupled to a transcriptional effector domain. Depending on the effector domain, gene expression can be transiently upregulated or downregulated (and even permanent modulation can be envisioned by targeting epigenetic enzymes to the promoter of interest). As ATF-mediated upregulation induces the expression of all splice variants in their natural ratios, ATF approaches mimic nature more closely as compared to conventional gene therapy where generally one cDNA is transferred into cells. With respect to downregulation, the ATF approach might be applied as a powerful alternative to (or in synergistic combination with) RNA targeting approaches as generally only two copies of the DNA have to be bound per cell as compared to the numerous mRNA molecules addressed by siRNA approaches.

To date, several different DNA sequence recognition modules have been developed, including triple-helix forming oligonucleotides (TFOs), synthetic polyamides and designer zinc finger proteins (1). The DNA binding domain in most ATFs is based on engineered zinc finger proteins (ZFPs), which can be designed to target unique sequences of 18 bp or longer, as described extensively in this volume. The expression of a number of endogenous genes has been modulated using ZFP-based ATFs (1). In vivo animal models further demonstrated therapeutic relevance of ATFs by studying the endogenous upregulation of *utrophin* (2) and *maspin* (3), as well as the downregulation of *VEGF-A* (4). In fact, convincing therapeutic effects in several animal studies performed by Sangamo Bioscience have allowed the initation of clinical trails (now at phase II) to test a three finger ZFP-based ATF, which targets 9 bp in the VEGF-A promotor, for its ability to induce therapeutic angiogenesis (5).

To specifically upregulate gene expression, transcriptional activation effector domains are fused to engineered ZFPs, such as the herpes simplex virus protein VP16 (or its tetramer VP64, which consists of four VP16 activation subunits). VP16 activates gene expression by facilitating the assembly of a RNA polymerase II preinitiation complex and interaction with chromatin-remodeling enzymes (e.g., components of the SWI/SNF complex). Alternatively, the NF-κB transcription factor p65 domain or b-catenin subunits can be used to recruit transcription promoting factors to genes of interest (6).

The most commonly used repressive effector domain in ZF-ATFs is the human Krüppel-associated box (KRAB) domain of the KOX1 protein. The KRAB domain consists of an A and B box and a stretch of 45 aa in the KRAB A box has been defined to function as repressor of transcription. Repression occurs by the recruitment of enzymes responsible for changing the epigenetic environment; the action of these enzymes interferes with the TATA box complex assembly and results in relocation of the gene to heterochromatin compartments in the nucleus. Other powerful tools with transcriptional repressive effects

are the mSin3 interaction domain (SID, which consists of 35 aa located in the N-terminus of the transcription factor Mad), the ligand dependent thyroid hormone receptor (TR) or its viral relative vErbA (6).

Unfortunately, at this point, no predictions can be distilled from the literature on which effector domain will work best for a given promoter. Although some studies suggest specific promoter characteristics for certain effector domains to function well, empirical data do not support any such strict rules. Similarly, no absolute rules for optimal target sequences within a promoter can be given, as one effector domain might work efficiently when targeted by a specific ZFP while another gives no effect when fused to the same ZFP (1). Apart from affecting the function of effector domains, the chromatin environment also affects the binding of the ZFP. Combining ATF approaches with chromatin opening drugs is thus likely to result in synergism, as recently shown for ATF-induced upregulation of the epigenetically silenced *maspin* gene (7).

In this chapter we present data on three and six finger ZFPs, constructed according to a modular design strategy (8) to target the promoter of Epithelial Cell Adhesion Molecule (EpCAM) (9) or of the RNA fragment of telomerase (hTR) (10). The hTR gene is an integral part of the telomerase holoenzyme, which is specifically expressed in the majority of cancer cells and directly confers immortality (11). Similar to the situation for EpCAM, levels of hTR are low in normal cells, but are substantially increased in cancer cells. Strategies to manipulate these expression levels are of substantial clinical and scientific interest in the cancer field.

Methods 3.1 and 3.2 describe cloning strategies to obtain prokaryotic as well as eukaryotic expression plasmids for ZFPs. Approaches to obtain purified ZFPs are described followed by assays to validate correct expression (western blot) and target DNA binding (electromobility shift assay). After validation, regulatory effector domains (eg VP64 or KRAB for up- or downregulation, respectively) are cloned up- or downstream of the ZFP in the eukaryotic expression plasmid. The resulting ATFs can be tested for functionality after transfection of the eukaryotic expression plasmid in living cells. In method 3.3 we describe co-transfection experiments with reporter luciferase plasmids, to assess the modulation of hTR promoter activity by luciferase assay. For surface proteins, the endogenous gene expression regulation can be visualised by flow cytometry analyses as described in this chapter for EpCAM.

2. MATERIALS

2.1 Construction of the eukaryotic expression plasmid for ATFs.

1. TE: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
2. Linker oligonucleotides to construct a eukaryotic ATF expression plasmid, as well as oligonucleotides to obtain the ZFPs and effector domains, as depicted in **Table 1** (*see* **Note 1**). Oligonucleotides are dissolved in TE.
3. Eukaryotic expression plasmid pcDNA3.1(+)C (Invitrogen).
4. Restriction enzymes: AgeI, BglII, HindIII, PmeI, XhoI, XmaI (New England Biolabs).
5. Mung bean nuclease (New England Biolabs).
6. T4-DNA ligase and calf intestinal alkaline phosphatase (Fermentas).
7. *E.coli* strain DH5a, Luria Broth base medium (Invitrogen), and ampicillin.

8. High fidelity Pfu Turbo polymerase (Stratagene).
9. Plasmid preparation kits, PCR product purification and gel extraction kits (Qiagen).

Table 1. Oligonucleotides

A	KOZAK upper	5′-agcttccgccatggttagatctcaaagaagaagagaaaagttaccggtggatcctc
	KOZAK lower	5′-tcgagaggatccaccggtaacttttctcttcttctttggagatctaaggatggcggga
B	Const F1-f	5′-gaggagggatccCCCGGGgagaagccctatgcttgtccggaatgtggtaagtccttcagc
	hTR-F1-f	5′-tgtggtaagtccttcagc**cataaaaatgcactgcaaaat**caccagcgtacccatacgg
	Const F2-r	5′- ctgaaagacttgccgcactctgggcatttatacggtttttcacccgtatgggtacgctgg
	hTR-F2-f	5′-gtgcggcaagtctttcagt**gattgtagagatctggcaaga**catcaacgcacccacactgg
	Const F3-r	5′-gagaaggacttgccacattctggacatttgtatggcttctcgccagtgtgggtgcgttg
	hTR-F3-f	5′-atgtggcaagtccttctct**agaagtgatgaactggtaaga**caccaacgtactcacaccg
	Term F3-r	5′ctcctcaagctttcaACCGGTgtgagtacgttggtg
	hTR-F4-f	5′-tgtggtaagtccttcagc**agaagtgatgaactggtaaga**caccagcgtacccatacgg
	hTR-F5-f	5′-gtgcggcaagtctttcagt**agaagtgataaactggtaaga**catcaacgcacccacactgg
	hTR-F6-f	5′-atgtggcaagtccttctct**caaagagcacatctggaaaga**caccaacgtactcacaccg
	Term F3-r LINKER	5′-ctcctcaagctttcaaccggt*gcttcctccgcttcctcc*gtgagtacgttggtg
C	KRAB: forward primer	5′-aacGGATCCagaacattagtaactttcaaagacgtattcgtagacttcacaagagaagaat Ggaaattattagacacagcacaa
	KRAB: reverse primer	5′-tttGGATCCtaagcttactaagtttttgtagttttctaacattacgtttctgtatactatttgttgtgct Gtgtctaataatt
D	VP64:forward primer	5′-aaaGGATCCgatgcattagatgactttgacttagatatgctaggatctgacgcgctagacga tttcgatctggacatgttgggcagcgatgctc
	VP64:reverse primer	5′-tttAGATCTcagcatgtcgaggtcgaagtcatccagggcatccgagccaagcatatctaaat cgaaatcgtccagagcatcgctgcccaacatg

Oligonucleotide sequences. Sequences of oligonucleotides required for constructing ZFPs and their ATFs. Primers are dissolved in TE to 1 µg/µL (work solution 1:20) and stored at -20°C unless stated differently. A) KOZAK-linker sequence to create the pcDNA3.1mnhKOZAK backbone; B) Primers for a 6-finger ZFP (F1-6) construct targeting hTR (nucleotides encoding amino acids -1 to 6 are shown in bold); C) Oligos for primer elongation of the downregulatory KRAB effector domain. Restriction sites are shown in capitals and complementary sequences are underlined; D) Oligos for primer elongation of the upregulatory VP64 effector domain. Restriction sites are shown in capitals and complementary sequences are underlined.

2.2 Protein studies

2.2.1 ZFP protein induction and purification (pGEX4T1-ZFP)

1. Prokaryotic expression plasmid pGEX4T1 vector, which contains a GST tag for protein purification (GE Healthcare).
2. *E.coli* strain BL21, 2YT medium (Invitrogen), and ampicillin.
3. Glucose.
4. Isopropyl-b-D-thiogalactopyranoside (IPTG, Invitrogen).
5. Binding Buffer: PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3).
6. Filter 0.45 µm (Corning).

7. Elution Buffer: 50 mM Tris-HCl, pH 8.0, 10 mM reduced gluthatione (Sigma).
8. GSTrap HP 1 mL columns (GE Healthcare). Store in 20% EtOH at 4°C.
9. FPLC system (e.g., ÄKTA Explorer, GE Healthcare).
10. French Press (Thermo).
11. 2x ZF Protein Storage Buffer: 20 mM Tris-HCl, pH 7.9, 10 mM DTT, 0.1 mM $ZnCl_2$, 2 M NaCl, 10% glycerol, 0.25x protease inhibitor cocktail.

2.2.2 Electromobility shift assay (EMSA)

1. Oligonucleotides: 20 bp containing the target sequence or non-target sequences. Only one strand of the target sequence needs to be labeled with IRdy681. For annealing of oligonucleotides, use 10x PCR buffer without Mg^{2+} (Fermentas).
2. Anti c-Myc antibody (mouse immunoglobulin, Sigma-Aldrich).
3. Gel casting system (Bio-Rad).
4. Gel analysis system (e.g., Odyssey Scanner, Li-Cor Biosciences).
5. TBE: 95.4 g Tris, 2.75 g boric acid, 2 mL 0.5 M EDTA, pH8, in 100 mL water.
6. Polyacrylamide gel materials: 40% acrylamide (acrylamide:bisacrylamide 29:1, Warning: toxic when soluble!) (Bio-Rad), N, N, N, N'-tetra-methyl-ethylenediamine (TEMED, Bio-rad), ammonium persulfate (APS, Pharmacia Biotech).
7. Loading Buffer: 20% Ficoll 400 (Qbiogene), bromophenol blue.
8. Binding Buffer: 100 mM HEPES, pH 7.5, 500 mM KCl, 10 mM DTT.
9. Polydeoxyinosinic-deoxycytidylic acid (poly dI-dC, Sigma).

2.2.3 Western Blot

1. 1° antibody: anti-c-Myc (*see* **Materials 2.2.2**); 2° antibody: anti mouse IgG peroxidase (DAKO Cytomation).
2. Blotting filters (Whatman paper, Whatman).
3. Nitrocellulose membrane (Whatman).
4. Luminol (SuperSignal West Dura Extended Duration Substrate, Pierce).
5. Polyacrylamide gel materials: 40% acrylamide, 0.5 M Tris-HCl, pH 6.8, 1.5 M Tris-HCl, pH 8.8, 10% Sodium-Dodecyl sulfate (SDS), TEMED, APS.
6. 2′ Electrophoresis Sample Buffer: 1 mL of 0.5 M Tris-HCl, pH 6.8, 1.6 mL of 10% SDS, 800 µL of glycerol, 400 µL of 1% bromophenol blue, 400 µL of b-merceptoethanol. Add 3.8 mL Milli-Q-purified water to 8 mL final volume.
7. 10′ Running Buffer: 30.3 g Tris (0.25 M final), 144 g glycine (1.92 M final) and 10 g SDS (1% final) in 1 L Milli-Q-purified water.
8. 10′ Blotting Buffer: 30.3 g Tris (0.25 M final) and 144 g glycine (1.92 M final) in 1 L Milli-Q-purified water. pH should be 8.3 *(Do not adjust).*
9. Blocking Buffer (make fresh): skim milk (5% in PBS) and Tween 0.1%.

2.3 ATF validation

2.3.1 Cell Culture and transfection

1. Cells with or without the expression of the gene of interest (ATCC).

2. 96-well tissue culture treated luminometer plates (Nunc, #136101).
3. Transfection reagent (e.g., Superfect (Qiagen), or SAINT-mix (Synvolux Therapeutics)).

2.3.2 Luciferase assay

1. Luciferase Stop and Glo assay kit (Promega).
2. Luciferase reporter plasmid containing the ZFP target sequence (e.g., pGL3hProm867, which contains part of the hTR gene) (10).
3. Renilla luciferase control plasmid pRL-SV40 (Promega).
4. Microplate luminometer (e.g., Lumicount, Packard).

2.3.3 Flow cytometry analysis

1. Flow cytometer (e.g., FACSCalibur, BD Bioscience).
2. 1° antibody: an EpCAM specific mouse monoclonal antibody (IgG1), produced by a hybridoma cell line and purified by protein A column chromatography (e.g., Prosep A high capacity, Millipore).
3. 2° antibody: polyclonal rabbit-anti-mouse $F(ab)_2$-FITC (DAKO).
4. Valproic acid (VPA, Sigma-Aldrich).

3. METHODS

3.1 Construction of the eukaryotic expression plasmid for ATFs (see Note 1)

3.1.1 Cloning of the backbone pcDNA3.1mnhKOZAK (see Note 2)

1. Anneal KOZAK oligonucleotides (*see* Table 1) by adding 5 nmol of each to a final volume of 50 µL of TE, incubate at 94°C for 1 min, lower the temperature by 2°C every cycle down to 20°C (cycles 2-36).
2. Digest pcDNA3.1(+)C with AgeI and PmeI (removing the AgeI site and the His-tag sequence) and blunt the overhangs using mung bean nuclease. Religate (*see* Note 3).
3. Digest the resulting pcDNA3.1mycnonhis plasmid with XhoI and HindIII and ligate with the annealed KOZAK linker to obtain pcDNA3.1mnhKOZAK.

3.1.2 Obtaining three finger ZFPs (7 primer method) (see Note 4)

1. Mix 50 ng of each primer (i, ii, iii, iv, v, vi and vii for F1-3; and i, viii, iii, ix, v, x, vii (or xi, *see* Note 5) for F4-6; Table 1) with 5 U high fidelity Pfu turbo polymerase, 0.4 µL of dNTPs (100 mM) and 5 µL of 10′ polymerase buffer, fill up to 50 µL with Milli-Q-purified water and run a PCR reaction (cycle 1: 5 min at 95°C; cycles 2-11: 30 s 94°C, 30 s 62°C, decrease annealing temperature 1°C each cycle, 30 s 72°C; cycles 12-30: 30 s 94°C, 30 s 52°C, 30 s 72°C; cycle 31: 5 min at 72°C; samples can be on hold at 4°C).
2. Purify the PCR product of 285 bp out of a 2% agarose gel, elute in 30 µL of H_2O and digest the product using XmaI and AgeI immediately before Method 3.1.3.

3.1.3 Cloning of six finger ZFP-ATF plasmids

1. Digest pcDNA3.1mnhKOZAK with AgeI, dephoshorylate and ligate to the digested ZFP-PCR product F1-3.

2. Digest resulting pcDNA3.1mnhK-F1-3 with AgeI, dephoshorylate and ligate to the digested ZFP-PCR product F4-6.
3. Obtain effector domains by primer extension of described oligonucleotides (*see* Table 1) and digest the obtained product using BamHI only (for KRAB = 135 bp) (*see* Note 6) or BamHI and BglII (for VP64 = 156 bp).
4. Digest pcDNA3.1mnhKOZAK-F1-6 with BamHI, dephosphorylate and ligate to digested effector domain fragment (*see* Notes 3 and 7).

3.2 In vitro ZFP protein studies

3.2.1 ZFP protein induction and purification (pGEX4T1-ZFP)

1. Digest pcDNA3.1mnhKOZAK-ZFP with BglII (*see* Note 6) and BamHI and ligate the ZFP-containing fragment to BamHI digested, dephosphorylated pGEX4T1. Transform into BL21 *E. coli*.
2. Use a correct clone of BL21 the transformants to inoculate a 6-mL starter culture in 2YT-medium supplemented with 10% glucose and 1% ampicillin overnight at 37°C.
3. Inoculate 500 mL of 2YT-medium with 0.1% glucose and 1% ampicillin using 5 mL of the starter culture. Grow at 37°C in the shaker until OD_{600} = 0.5 (about 2.5 h).
4. Add IPTG in a final concentration of 0.5 µM to induce protein production.
5. Grow for 4 h at 37°C in the shaker.
6. Harvest by centrifugation at 4000 ' g for 30 min at 4°C.
7. Freeze the pellet at -20°C (can be stored at -20°C until further use).
8. Resuspend the pellet in 10 mL of PBS (*see* Note 8).
9. Use a French Press (3 ' at 2500 psi) to squeeze the bacteria suspension.
10. Centrifuge the bacterial lysate for 15 min at 2300 ' g.
11. Filter supernatant through a 0.45-µm filter.
12. Bind and elute the protein as described by the equipment manufacturer (e.g., using GSTrap HP 1-mL columns on an ÄKTA Explorer).
13. Store the protein eluates in 1x ZF Protein Storage Buffer at -80°C

3.2.2 Electromobility Shift Assay (EMSA)

1. Dissolve each oligonucleotide in TE, pH 8, to 100 µM (store at -20°C).
2. Anneal upper and lower oligonucleotides (keep dark) by mixing 2.5 µL of sense oligonucleotide, 2.5 µL of anti-sense oligonucleotide, 5 µL of 10' PCR Buffer, and 40 µL of Milli-Q-purified water. Boil for 5 min at 100°C and slowly cool it to room temperature by allowing the boiling water to cool down. Store at -20°C.
3. Prepare a 4% polyacrylamide gel by mixing 0.75 mL of 5' TBE, 0.75 mL of 40% acrylamide, 6 mL of Milli-Q-purified water, 4.5 µL of TEMED and 37.5 µL of APS. Pipet carefully between the glass plates until you get a little overflow.
4. Place the combs. Let the gel polymerize for 45 min. Pre-run the gel in 0.5' TBE for 30 – 60 min at 100 V.
5. For a supershift, first incubate 10 ng of purified ZFP (*see* Note 9) 1:1 (w:w) with anti c-Myc antibody for 30 min at room temperature.

6. Preparation of the samples: Prepare a master mix for all tubes to contain 2 µL of 10′ Binding Buffer and 1 µL of poly dI:dC. Add the appropriate amount of Milli-Q-purified water for a final volume of 20 µL. Subsequently, add the appropriate amount of cold probe for competition experiments (we generally use 50′ and 100′ excess (1 µL = 10 pmol, and 2 µL = 20 pmol, respectively). Add 10 ng of purified ZFP to all tubes except for the first (probe only tube). Always add hot probe last (2 µL of a 1:100 dilution = 0.2 pmol).
7. Incubate reaction tubes for 20 min at room temperature. Add 7 µL of Loading Buffer and load 20 µL on the polyacrylamide gel.
8. Run the gel for 30 min at 100 V and analyze on the Odyssey Scanner.

3.2.3 Western Blot

1. Prepare a 12% acrylamide solution for the separating gel by mixing 3 mL of 40% acrylamide, 2.5 mL of 1.5 M Tris-HCl, pH 8.8, 100 µL of 10% SDS, 4.35 mL of distilled deionized water. Add 5 µL of TEMED and 50 µL of 10% APS and carefully pipette between the glass plates. Overlay with isobutanol to have a straight surface.
2. Prepare a 4% stacking gel by mixing 1 mL of 40% acrylamide, 2.5 mL of 0.5M Tris-HCl, pH6.8, 100 µL of 10% SDS, and 6.33 mL of distilled deionized water.
3. When the 12% separating gel is polymerized, remove the layer of isobutanol and add 10 µL of TEMED and 50 µL of 10% APS to the 4% acrylamide solution. Pour the stacking gel carefully on the separating gel and place the comb.
4. Dilute your samples 1:2 in 2′ Electrophoresis Sample Buffer.
5. Heat samples to 95°C for 3-5 min (i.e., in boiling water).
6. Load the samples and a protein marker.
7. Electrophorese the samples in 1′ Running Buffer. Set the generator on 80 V until the samples have penetrated the stacking gel (15-30 min), then go to 200 V until the colored front of the loading buffer has reached the bottom of the gel.
8. For blotting, prepare a sandwich by placing a soaked blotting sponge, a soaked Whatman filter, the gel, a soaked nitrocellulose membrane, a soaked Whatman filter and a soaked blotting sponge in the cassette. All are soaked in 1′ Blotting Buffer.
9. Insert the cassette in a blotting chamber and add pre-chilled 1′ Blotting Buffer.
10. Transfer at 100 V for 1 h.
11. When finished immerse the membrane in Blocking Buffer, and incubate overnight at 4°C.
12. Add the primary anti-c-Myc antibody to the Blocking Buffer.
13. Wash the membrane three times for 10 min with PBS containing 0.1% Tween.
14. Incubate with the secondary antibody: anti-mouse IgG peroxidase in blocking buffer.
15. Wash four times 10 min with PBS containing 0.1% Tween.
16. Develop the membrane by incubating for 5 min with equal parts of the Stable Peroxide Solution and the Luminol Enhancer Solution according to manufacturer (0.1 ml per cm^2). Cover the blot with a clear plastic wrap and expose to X-ray film.

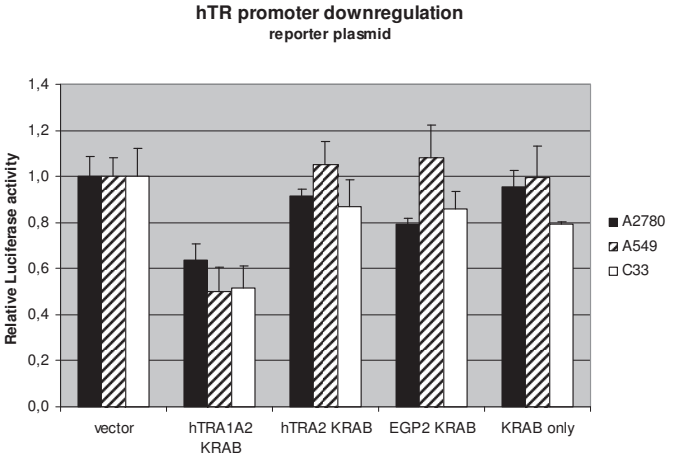

Figure 1. Luciferase activity assays for ZFP-based ATFs. The graph shows luciferase activity following the co-transfection of ATF plasmids with a reporter plasmid containing the luciferase gene under the control of a fragment of the hTR promoter. The ATF plasmids contain a 6-finger ZFP directed against hTR (A1A2), a 3-finger ZFP against hTR (A2), or a 3-finger ZFP against EpCAM (EGP2), all fused to the KRAB repressor domain. The pcDNA3.1mnhKOZAK KRAB plasmid containing an untargeted KRAB domain only (KRAB) was also included. Co-transfections were performed in three different hTR positive cell lines (ovarian cancer A2780, lung cancer A549 and cervical cancer C33). Results are the mean of the mean of three independent experiments performed in triplicate. Inhibition of hTR promoter activity was up to 50% compared to the vector-only control; p= 0.02 for all three cell lines. No effect was seen on three irrelevant promoters (data not shown).

3.3. Cell-based validation of ATFs

3.3.1. Co-transfection using reporter plasmids (see Fig. 1)

Co-transfection and luciferase assay are performed in a single vessel format using 96-well luminometer plates.

1. Seed cells in a volume of 200 mL into triplicate wells of 96-well luminometer plates 24 h prior to transfection, at a density sufficient give 70-90% confluence at the time of transfection (*see* **Note 10**). For the cell lines used in **Fig. 1**, seed 5 ' 10^4 cells per well.
2. Appropriate controls for each assay include: a promoter reporter of interest, a Renilla luciferase expression vector for normalization of firefly luciferase activity, each ATF expression construct of interest, an ATF construct not specific for the promoter of interest, a vector-only control, an effector domain-only control. It may also be desirable to include mutant promoter constructs modified at the predicted ATF binding sites.
3. For each well to be transfected, dilute 250 ng of firefly luciferase expression vector containing promoter of interest along with 125 ng of ATF expression construct and 30 ng of Renilla luciferase expression construct in 30 mL of serum-free growth medium (*see* **Note 11**). For triplicates, make a 4' DNA Master Mix for each transfection condition.

4. For each well to be transfected, dilute 0.625 mL of Superfect transfection reagent in 20 mL of serum-free growth medium. Make a Superfect Master Mix sufficient for all wells (*see* Note 12).
5. Add 80 µL of Superfect Master Mix to each 120 µL of 4′ DNA Master Mix and incubate at room temperature for 15 min.
6. To each tube containing the complex of Superfect and DNA , add 600 µL of complete growth medium.
7. Remove the cells from incubation. Aspirate the growth medium from each well using, for example, a 16-gauge syringe needle. Rinse each well once with PBS. Aspirate the PBS.
8. Add 200 µL of the medium containing Superfect and DNA to triplicate wells and return the cells to incubation for 2.5 h.
9. Aspirate the transfection complexes from each well and rinse once with PBS. Remove the PBS and add back 200 µL of complete growth medium to each well.
10. Incubate the cells for 48 h prior to the luciferase assay.

3.3.2 Luciferase assay (see Note 10)

1. Aspirate the medium from all wells using, for example, a 16-gauge syringe needle attached to a vacuum line. Aspirate the medium from the edge of the well to avoid damage to the cell layer.
2. Using a multi-channel pipette, rinse the wells once with 200 µL of PBS.
3. Aspirate the PBS as described for medium in Method 3.3.1.
4. To each well, add 50 µL of 1′ Passive Lysis Buffer, diluted with distilled water from the 5′ solution provided in the Stop and Glo assay kit.
5. Incubate at room temperature for 20 min with gentle shaking using, for example, a rocking table or vortexer with microplate attachment on low speed.
6. Read the luciferase activities of the control and ATF-transfected wells using a microplate luminometer. 50 µL of Luciferase Assay Substrate dissolved in Luciferase Assay Buffer II is added to each well, followed by 50 µL of Stop and Glo Substrate dissolved in Stop and Glo Buffer. Measure each luciferase activity over 5 s with a 2-s delay following injection of each reagent.
7. Normalize the firefly luciferase activity to the Renilla activity of each well using the formula $L_N = L_W' (R_M / R_W)$, which preserves the magnitude of the firefly luciferase activity (L_N = normalized firefly luciferase activity, L_W = firefly luciferase activity of a well, R_M = mean Renilla activity of the plate, and R_W = Renilla activity of the well).
8. Calculate the mean and standard errors of triplicate wells using appropriate software (e.g., Microsoft Excel).
9. The relative effect of each ATF on a promoter of interest, such as promoter repression, is estimated by determining the relative luciferase activity: divide the mean luciferase activity of triplicate ATF transfectants by that of vector-only transfectants (*see* Fig. 1). The transfection/luciferase assay should be repeated at least three times.

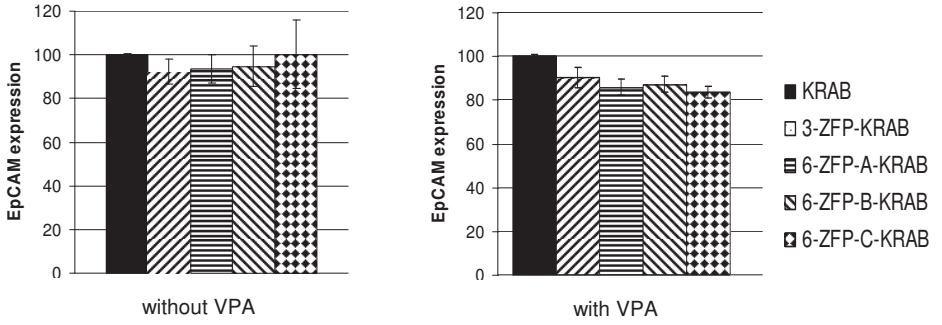

Figure 2. Downregulation of the endogenous EpCAM promoter. SKOV-3 cells were transfected with ATF expression plasmids (described in ref *9*) in the presence or absence of the chromatin opener VPA. No effect on endogenous promoter activity could be observed in the absence of VPA. However, the combinatorial treatment of ATF-constructs and VPA resulted in a slight downregulation of up to 15% of the endogenous EpCAM expression as measured with flow cytometry. Results are the mean of the mean of three independent experiments performed in duplo (* p< 0.05; ** p< 0.01; *** p< 0.001)

3.3.3 Flow cytometry analysis of targeting the endogenous promotor (EpCAM) (see Fig. 2)

1. Plate the cells at a density of e.g. 250,000 cells/well in a 6-well plate (*see* Note 10).
2. Transfect the cells at a confluency of about 70% (*see* Note 12).
3. Culture the cells for 72 h at 5% CO_2 and 37°C, with or without VPA (*see* Note 13).
4. Harvest the cells using TEP (Trypsin/EDTA/PBS) (*see* Note 14).
5. Wash cells two times with 2 mL of PBS, and centrifuge 5 min at 500 ' g at 4°C.
6. Add 100 µL of MOC-31 supernatant, resuspend the cells and incubate for 1 h at 4°C.
7. Wash twice with 2 mL of PBS, and centrifuge 5 min at 500 ' g at 4°C.
8. Aspirate the supernatant and add 100 µL of polyclonal rabbit-anti-mouse Ig-FITC antibody diluted 1:40 in 5% pool serum. Resuspend.
9. Incubate for 30 min at 4°C in the dark.
10. Wash two times with 2 mL of PBS, and centrifuge 5 min at 500 ' g at 4°C.
11. Aspirate the supernatant and resuspend the pellet in 200 µL of PBS.
12. Measure on the FACSCalibur 100,000 cells. Analyze the results using WinList software.

4. NOTES

1. All plasmids described in this chapter will be provided by the authors upon request.
2. Linkers can be designed to contain any choice of restriction sites. The described KOZAK linker contains the Kozak sequence (underlined in Table 1), followed by a BglII restriction site (*see* Note 6), a nuclear localization signal, AgeI and BamHI sites, and one additional nucleotide to keep the reading frame for Myc tag expression. After annealing, the linker is flanked with overhanging HindIII and XhoI sites.
3. To monitor unwanted effects of mung bean nuclease on double stranded DNA, always sequence your plasmid before continuation. Obviously, all further described primer

elongation products and similar materials should also be verified by sequencing analysis.

4. Please refer to reference (8) for further information on the 7-primer method, as well as other relevant chapters in this volume regarding ZFP design. Check for the absence of BamHI restriction sites when designing the ZFPs. Always construct several ZFPs for targeting a specific promoter: For EpCAM, we constructed one 3F ZFP and two 6F ZFPs, all of which worked nicely in reporter studies (9). In contrast, for hTR we constructed five 3F and two 6F ZFPs, for which only one of the 6F ZFP worked consistently.
5. To improve flexibility within the ATF, include a GGSGGS-linker in the reverse primer of the last finger (F3 in three-finger ZFPs or F6 in six-finger ZFPs) (*see* Table 1, primer xi).
6. We created a HindIII site in the KRAB construct to verify orientation. VP64 can be checked using BamHI. Obviously, other restriction enzyme recognition sites can be easily included in the linker as well as in the effector domains to use your favorite restriction enzyme. You might also want to clone the effector domain upstream of the ZFP by using BglII partial digestion (BglII is also present in the pcDNA3.1 backbone 904 bp 5′ of the BglII site in the linker).
7. Also, construct plasmids containing the effector domain only as controls.
8. DTT (1-10 mM) can be added to the binding and elution buffer to increase binding of the protein to the column.
9. Always test several amounts of your purified ZFP first for binding to the probe as affinities differ for different ZFPs.
10. Appropriate cell seeding densities should be determined for the culture area and cell type prior to performing the transfection. In the 96-well white luminometer plate format, confluence cannot be examined microscopically prior to transfection. Therefore, determine appropriate seeding densities by seeding a range of concentrations in clear 96-well plates before performing the transfection in luminometer plates. It is also common to perform transfections and harvesting in a clear vessel format, then transfer cell lysates to luminometer plates for luciferase assay. In the single vessel format, inclusion of renilla luciferase provides a control for transfection efficiency and minor variations in well to well densities.
11. Optimization of plasmid vector amounts may be required in different cells. 30 ng pRL-SV40 provides sufficient signal for normalization in the cells used here. To determine optimal amounts of vectors, titration of the ATF and Renilla constructs should be performed, using empty vector to keep the total DNA amount constant.
12. The optimal ratio of Superfect to plasmid DNA varies according to the cell line. The amount of Superfect stated in the method has been used to successfully transfect several cell lines, including the C33A, A2780 and A549 cells shown in Fig. 1. However, optimization of Superfect volume may be necessary for other cell lines. Also other transfection agents can be used (SAINT transfections have been performed for the FACS experiments).
13. Other epigenetic drugs can be used to open up the chromatin. Always determine the optimal, non-toxic dose for each cell line.
14. Include positive and negative cells as control, also incubate a control with conjugated 2° antibody only and include an unstained sample to exclude autofluorescence.

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Chapter 3

Targeting the Epithelial Cell Adhesion Molecule (EpCAM) using Zinc Finger-based Artificial Transcription Factors

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ABSTRACT

In most carcinomas the expression of the Epithelial Cell Adhesion Molecule (*EpCAM*) is dysregulated and the correction of EpCAM expression in these cancers types could be of therapeutic benefit. To efficiently change gene expression, Zinc Finger proteins (ZFPs) have been used to target virtually any gene of interest. Fused to the repressor domain KOX1 (SKD) or the trans-activator domain VP64, ZFPs can down- and upregulate endogenous gene expression, respectively. Here we set out to screen a retroviral ZF library consisting of 2 x 10^7 different 6-finger ZFPs. We delivered the ZFP library into human cancer cells and used fluorescence activated cell sorting to isolate cells with modulated EpCAM gene expression. The ZFP DNA was recovered and subcloned in the ATF-VP64-library to identify positive regulators of EpCAM gene expression, however, the cell based screening of the ZFP library did not result in EpCAM modulating ZFPs. In parallel, previously designed ATFs, which showed regulatory activity on the exogenous but not the endogenous EpCAM promoter were subcloned into a retroviral expression system and tested for their regulatory efficiency to regulate the endogenous promoter after retroviral delivery.

INTRODUCTION

The Epithelial Cell Adhesion Molecule (*EpCAM*) initially described as a tumor-associated antigen, has a versatile role in cell differentiation, proliferation and migration (1,2). A close functional link was unravelled between *EpCAM* and the activation of the Wnt-signalling pathway, which is known to play a pivotal role in cancer, embryogenesis and maintenance of stem cell properties (3-5). Furthermore, *EpCAM* expression was found in tumor initiating/cancer stem cells of colon, breast, liver and pancreatic cancer (6-9). Therefore, *EpCAM* attracts notice as 1) stem cell marker involved in self-renewal and 2) marker expressed on cancer (stem) cells in tumours of variable origin (10). However, in the context of cancer, *EpCAM* expression is controversial: While its upregulation in some cancer types is correlated with a positive prognosis, e.g. in colon and renal carcinomas, in others *EpCAM* overexpression is associated with a poor prognosis, e.g. breast, gall bladder and cervix carcinoma (reviewed in (2)). To investigate the versatile role of *EpCAM* in different cancer types, we choose *EpCAM* as a target for transcriptional up- and downregulation.

Regulation of gene expression directly on DNA level can be achieved by Artificial Transcription Factors (ATFs). At minimum, ATFs consist of 1) a DNA binding domain (DBD) designed to specifically bind a desired DNA sequence and 2) an effector domain (ED) for transcriptional regulation. In the majority of ATFs, engineered Zinc Finger Proteins (ZFPs) are exploited as DNA binding domains. A single ZF domain consists of approximately 30 amino acids, wherein a stretch of seven amino acids in the alpha-helix recognizes three to four base pairs in the major groove of double stranded DNA. ZFPs can be easily generated by PCR using constant and variable primers, in which the variable primers are coding for the recognition helices. Therefore, the recognition site of the ZFPs can be easily changed and fingers with almost any specificity can be engineered. The modular character of ZFPs allows the tandem array of individual ZF motifs, leading in theory to an increased specificity for the target sequence in the genome (11). To date, a number of endogenous genes have been either up- or downregulated using ATFs (12), which can facilitate the activation and repression of all natural isoforms of the gene of interest, whereas conventional methods using siRNA or cDNA in general target only one isoform.

We previously designed four ZFPs, three 6-fingers and one 3-finger ZFPs targeting the *EpCAM* promoter. All three ZFPs when fused to transcriptional modulators regulated efficiently the exogenous *EpCAM* promoter of a reporter plasmid (13). However, these rational designed ZFPs had no efficient regulatory effect on the endogenous *EpCAM*-promoter (14). Inefficient transcriptional modulation by ATFs on endogenous genes can have multiple reasons, e.g. the ZFPs cannot bind their target region in the chromatin context as the chromatin topology of the endogenous target site can influence endogenous ZFP binding. The accessibility of ATFs to their endogenous target region underlies epigenetic restrictions, including DNA methylation, histone modifications and nucleosome positioning. Also, rational designed ATFs might lack binding to the target sequence because of competition with natural transcription factors, and lack of regulatory performance can be elucidated by insufficient activity of the effector domain at a certain position in the genome or inefficient delivery of the ATF into a cell population (15).

In order to identify ATFs with the potential to regulate endogenous gene expression, libraries of ZFPs have been generated and the delivery of such ATF-pools has been carried out in various systems such as bacteria (16), yeast (17), mammalian cells (18) and plant cells (19). After delivery of an ATF library into a cell population, global changes in gene expression are evoked and a variety of pathways are altered to generate specific phenotypes. This allows for the selection of cells which acquired a desired phenotype and the subsequent isolation of the causing ZFPs.

To isolate ZFPs able to modulate endogenous *EpCAM* expression, we exploited a retroviral ATF library consisting of $2x10^7$ ZFPs linked to the repressor domain SKD (Krueppel-associated box domain) or the activating effector domain VP64. In a first round of selection, we delivered the ATF library linked to SKD into an *EpCAM* expressing cell line, followed by fluorescence activated cell sorting, selecting only cells that acquired repressed *EpCAM* expression on their cell surface. The isolated ZFPs were then fused to the SKD and VP64 domain and used for subsequent rounds of selection in an *EpCAM*-positive and an *EpCAM*-negative cell line, respectively. In agreement with previous findings reporting a low success rate when isolating ZFPs from an ATF library in mammalian cells (18), the cell based screening for ZFPs modulating endogenous *EpCAM* expression did not result in potent ZFPs. In addition, we used our previously by modular design generated ZFPs (13) to evaluate their activity at the endogenous *EpCAM* promoter after retroviral delivery. Therefore, the ZFPs were subcloned into the retroviral expression vector pMX-VP64 and pMX-SKD. Upon retroviral transduction of *EpCAM* negative and *EpCAM* positive cell lines one of the modular designed ZFPs showed a strong upregulation of EpCAM expression in EpCAM negative cells. The preliminary findings of the present study were basis of a study evaluating the functional role of EpCAM in ovarian cancer and resulted in a recent publication by van der Gun *et al*. (20).

Identification of potent regulators of endogenous *EpCAM* gene expression provides insight in the biological role of *EpCAM*. Moreover, modulation of *EpCAM* expression, which is described as a stem cell marker on colon, breast, pancreatic and liver carcinomas, might be used as a therapeutic approach in impede tumour initiation.

MATERIALS AND METHODS

Cell lines and cell culture

The human embryonic kidney retrovirus packaging cell line 293-GagPol and the breast cancer cell line MDA-MB231 (*EpCAM* negative) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin. The breast cancer cell line MCF7 (*EpCAM* positive) was cultured in Minimum Essential Media (MEM) supplemented with 1.5 g/L Sodium Bicarbonat, 0.1 mM Nonessential Amino Acid Solution (NEAA), 1mM Sodium Pyruvat, 0.01 mg/mL Bovine Insulin, 10% FBS and 1% Pen/Strep. The inflammatory breast cancer cell line SUM149, which consists of three *EpCAM* expressing subpopulations (*EpCAM*-negative, *EpCAM*-low and *EpCAM*-high), was cultured in HuMEC Basal Serum Free Media supplemented with 5% FBS, 1% Pen/Strep, Supplemental Bull Kit and 25 mg Bovine Pituitary Extract (GIBCO). The breast cancer

cell line MDA-MB435S was cultured in DMEM supplemented with 10% FBS, 1% Pen/Strep and 10 µg/mL bovine insulin. The Human Mammary Epithelial Cell Line SUM159 (EpCAM negative) was cultured in Ham's F-12 medium, supplemented with 5% FBS, 10 mM HEPES, 5 µg/mL insulin, and 1 µg/mL hydrocortisone. The EpCAM-high expressing ovarian cancer cell lines SKOV-3 and OvCAR were cultured in DMEM with 10% FBS supplemented with 200 mM glutamine, 30 mg gentamycine. The lung cancer cell line A549 was cultured in F-12K media supplemented with 10% FBS and 1% Pen/Strep. All cells were obtained from ATCC (Manassas, VA, USA) and maintained at 37° C and 5% CO2.

Identification of ZFPs regulating endogenous EpCAM gene expression from an ATF-Library

The retroviral ZF-library pMX-IRES-GFP-6F-GNN-SKD and pMX-IRES-GFP-6F-GNN-VP64 was generated as described previously (21). 293-GagPol packaging cells were plated in a density of 7 x 10^6 on a Polylysin-coated 15 cm^2 dish, respectively. Retroviruses expressing 2 x 10^7 ATFs were obtained by co-transfection of the 293-GagPol cells with 11.25 µg pMX library retroviral vector and 3.75 µg pMD.G plasmid using Lipofectamin PLUS transfection reagents as recommended by the manufacturer (Invitrogen). Secreted virus was cleared 48 hours post-transfection and used to infect 2.1 x 10^7 SUM149 cells. Infections were performed in total 4 times every 8 to 12 hours and cells were harvested 72 hours post-infection for RNA extraction (Qiagen, RNeasy kit) or EpCAM staining using PE-labelled EpCAM specific antibodies (BioLegend). Cells were sorted based on GFP expression and EpCAM modulation using a Becton Dickinson flow cytometer and subcultured for two weeks. Sorted cells were used for isolation of genomic DNA carrying the integrated ATFs. From isolated genomic DNA, the putative *EpCAM* promoter binding ZFPs were recovered using specific primers (5'-AGC CAG ATG TGA TCC TCC GGT TGG-3' and 5'-CAGAATTTCGACCACTGTGC-3'). The purified PCR product was subsequently re-cloned into the retroviral vectors pMX-SKD and pMX-VP64. These putative *EpCAM* regulating ATFs were then used for another cycle of infection and selection. Finally, individual ZFPs were isolated, recloned and sequenced. Then, their efficiency in regulating *EpCAM* expression was tested in the *EpCAM* high expressing cell line MCF7 for downregulation or in the *EpCAM* low expressing cell lines MDA-MB231 and SUM159 for upregulation of endogenous *EpCAM*.

Fluorescence Activated Cell Sorting (FACS)

Anti-EpCAM antibody-staining was performed 72 hours post-infection and post-transfection, respectively. Infected host cells were harvested using trypsin, followed by two PBS-washing steps. Approximately 2x10^5 host cells were incubated with 1:25 PBS-diluted PE-conjugated anti-EpCAM antibody in a volume of 100 µl per sample for 30 min in the dark. Transfected host cells were incubated with mouse MOC-31 anti-EpCAM antibody for one hour on ice, and then washed two times with ice-cold PBS. After washing cells were incubated on ice in the dark for one hour with a PE-conjugated anti-mouse IgG antibody. After incubation, cells were washed two times with 2 ml PBS and resuspended in 300 µl PBS + 2% FBS and examined using Becton Dickinson Flow Cytometer and Cell Quest Software.

Cloning pre-existing ZFPs into the retrovial vector pMX

The 3-finger ZFP EGP2, and the two 6-finger ZFPs ZFP-A and ZFP-B (Fig. 1) were previously designed, generated and in vitro tested in our laboratory (13). The ZFPs were sub-cloned into the retroviral vectors pMX-IRES-GFP-SKD (referred to as pMX-SKD) and pMX-IRES-GFP-VP64 vector (referred to as pMX-VP64) (18). Towards this end, three primer pairs were designed containing different flanking *SfiI*-digestion sites. After PCR amplification using *Pfu* polymerase (Promega, USA), ZFPs were *SfiI*-digested; gel purified using Qiagen gel purification kit and sub-cloned into the *SfiI*-site of pMX-SKD and pMX-VP64, respectively. Correct sequence was confirmed by sequencing. The obtained plasmids were subsequently used for virus generation as described below.

Transfection, Retrovirus Generation and Infection

Using SAINT (Synvolux, Groningen, NL) transfection reagent the EpCAM-high expressing cell lines SKOV-3 and OvCAR were transfected with rational designed ZFPs expressed from the pcDNA3.1 expression vector. Transfections were performed in 6 wells plates with 1 µg plasmid DNA coding for the ATFs, empty vector or SKD-only control according to manufactures instructions.

The rational designed ZFPs were cloned into the pMX-SKD and pMX-VP64, and plasmids were amplified by transforming electrocompetent TOP10 cells (Invitrogen) and purified (Qiagen, DNA purification kit) for following plasmid transfection. The Packaging cell line 293-GagPol was plated in a density of 3 x 10^6 cells on a Polylysin-coated 10 cm^2 dish (Corning) and 24 hours after seeding, the cells were co-transfected with 3.75 µg pMX retroviral vector and 1.25 µg pMD.G plasmid using Lipofectamin PLUS transfection reagents as recommended by the manufacturer (Invitrogen). Virus-containing supernatant of the GagPol cells was harvested 48 hours after transfection, cleared by filtration through a 0.22 µm filter (Millipore), supplemented with 8 µg/mL polybrene and applied for 8 to 12 hours to 1 – 3 x 10^5 host cells, plated one day before transduction. Infection was repeated two to four times, depending on the host cell line and host cells were harvested 72 hours after last infection for FACS, protein and RNA extraction.

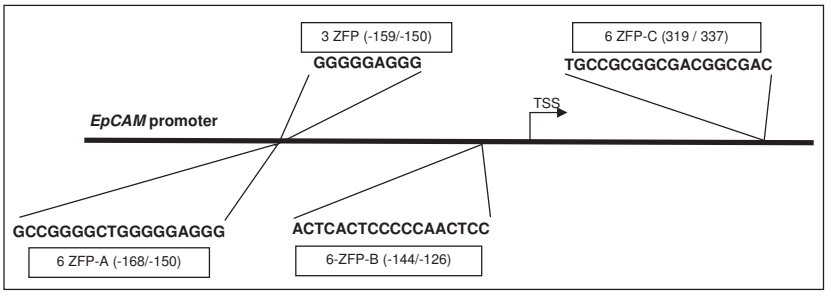

Figure 1. Figure 1 shows a schematic diagram of the *EpCAM* promoter with its transcriptional start site (TSS) and the target sequences of the four rational designed ZFPs relative to the TSS. Boxed numbers indicate distance to the TSS. In blue: 15 bp targeting ATF, green: 9 bp targeting ATF, black: 18 bp targeting ATF1, red: 18 bp targeting ATF ZFP-B-VP64.

cDNA conversion and qRT-PCR

Host cells were harvested 72 hours post-infection using 350 µl RLT Buffer (Qiagen) and either stored at -80°C until further processing or immediately used for RNA isolation according to manufacturer's recommendation. A total of 3 µg RNA was converted into cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems). Quantification of *EpCAM* and endogenous *GAPDH* control mRNA was measured by quatitative real-time PCR using Absolute Blue SYBR Green Low ROX Mix (Thermo Scientific). For *EpCAM* transcript amplification forward primer 5'-CTCGCGTTCGGGCTTCT-3' and reverse primer 5'-TGTAGTTTTCACAGACACATTCTTCCT-3' were used under the following PCR conditions: one initial step of 95°C for 15 min, followed by 40 cycles of 15 sec at 95°C, 30 sec at 58°C and 30 sec for 72°C. As an internal control *GAPDH* expression was used (primer sequences: Forward 5'-ACGGATTTGGTCGTATTGGG-3' and reverse 5'-TGATTTTGGAGGGATCTCGC-3'). TaqMan primer/probe mix (Applied Biosystems) was used for the detection of mRNA transcript of *SOX2* (ref #: Hs01053049_s1), *KLF4* (ref #: Hs00358836_m1, *KLF5* (ref #: Hs00156145_m1), *OCT4* (ref #: Hs03005111_g1) and *NANOG* (ref #: Hs02387400_g1).

Soft Agar Assay

To examine anchorage-independent growth of ATF-infected MDA-MB435S cells, Soft Agar analysis was performed. Per well of a 6-wells plate 2 ml of 0.6% agarose dissolved in cell culture media was plated to solidify. Cells were harvested 48 hrs post-infection and 5 x 10^3 cells were seeded in 2 ml of 0.3% agarose and plated on top of the 0.6% agarose bottom layer in the 6-wells plates. Cells were kept in a 37°C incubator humidified with 5% CO2. After 50 days, colonies were counted.

Cell Viability Assay

For cell viability analysis MDA-MB435S cells were harvested 48 hrs post-infection and plated in a density of 500 cells per well in 96-wells plate. Cell viability was measured at different time points by ATP-release using Cell Titer Glow (Promega) detected with PHERAstar detector (BMG Labtech, Cary, USA) and analysed using SPHERAstar software.

Statistic

For statistical analysis students t-test was carried out.

RESULTS

Transient transfection of rational designed ZFPs to target the EpCAM promoter does not lead to downregulation of EpCAM expression

We investigated the capability of four rational designed ZFPs (13) to downregulate endogenous *EpCAM* expression after transient transfection in the ovarian cancer cell lines SKOV-3 and OVCAR. Cells were transiently transfected using the expression vector pcDNA3.1 carrying the coding sequence for each ZFP. As controls empty pcDNA3.1 vector or pcDNA3.1 expressing the effector domain SKD was used. Subsequently surface EpCAM expression was detected by FACS analysis using an EpCAM-specific PE-labeled antibody.

Analysis showed no alteration of EpCAM expression after ZFP transfection when compared to empty vector and SKD control (Fig. 2).

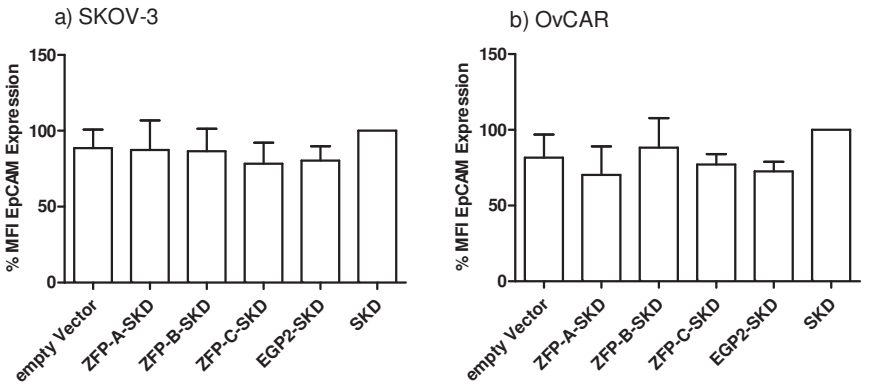

Figure 2. The ovarian cancer cell lines SKOV-3 (a) and OvCAR (b) were transiently transfected with four different EpCAM targeting ZF-ATFs and the EpCAM expression was detected by FACS.

Screening of an ATF-Library against EpCAM did not result in an enrichment of EpCAM-regulating ZFPs

To obtain *EpCAM* regulating ZFPs, an ATF-Library harbouring $2x10^7$ 6-finger ZFPs recognizing $(GNN)_6$ triplets fused to the transcriptional repressor SKD was screened in SUM149 cells with moderate *EpCAM* expression. Upon ATF-Library delivery, 2×10^5 cells were sorted based on high GFP and low EpCAM expression (Fig. 3a). Sorted cells were subcultured until enough cells could be obtained for genomic DNA extraction. Subsequently, DNA potentially coding for *EpCAM* targeting ZFPs was PCR-amplified and re-cloned into the retroviral expression vectors to obtain a smaller candidate-library of *EpCAM*-targeting ZFPs.

In a second cycle of ATF selection, the pool of ZFPs from the first selection round subcloned in pMX-SKD was used to infect SUM149 cells. $2.1x10^4$ cells with downregulated EpCAM expression were sorted (Fig. 3b). After cell sub-culturing, for 30 days no ZFP DNA could be retrieved from these cells. Additionally, the *EpCAM* positive ovarian cancer cell line SKOV-3 was subjected to retroviral ZF-library delivery to identify *EpCAM* downregulating ZFPs. Due to a low infection efficiency of SKOV-3 cells, sorting for cells with downregulated EpCAM expression was not possible (data not shown). In parallel, ZFPs from the first selection round were subclones into pMX-VP64 and delivered into the EpCAM-negative cell line SUM159 to screen for EpCAM upregulating ZFPs. Although SUM159 was infected with high efficiency (Fig. 3c), only a total of 389 cells with high EpCAM expression were isolated (Fig. 3c). Sorted cells were sub-cultured for 30 days; DNA putative coding for ZFPs was recovered from these cells and subcloned into pMX vectors. This resulted in 3 colonies upon transformation of bacteria. However, sequencing of these clones showed that no ZFP could be isolated after the ZF library screening.

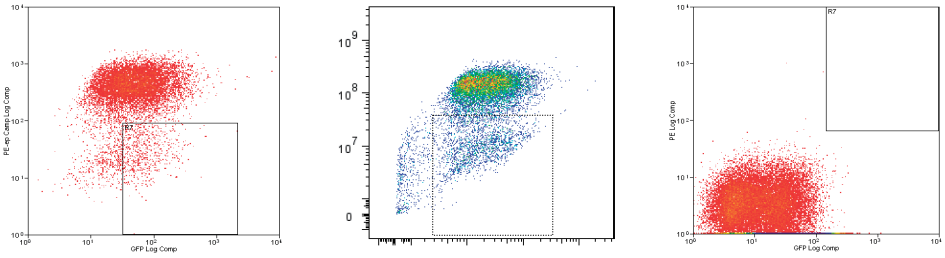

Figure 3. Cell sorting was performed on pMX-library-VP64/SKD-IRES-GFP-infected cells. 3a) First cycle of selection: SUM149 cells transduced with the $2x10^7$ ZFP-SKD-library. Gate marks $2x10^5$ sorted cells, positive for GFP and downregulated EpCAM expression. 3b) A second cycle of selection in SUM149 cells. PCR on selected cells did not result in isolation of ZFPs. 3c) Second cycle of selection in SUM159 cells for ZFPs to upregulate EpCAM expression. Gate marks 385 sorted cells, positive for GFP with upregulated EpCAM expression. Cells were subsequently used to recover DNA coding for putative EpCAM regulating ZFPs.

Modular designed ZFPs in the retroviral expression system indicates ATFs can modulate EpCAM expression

Next to the ATF library screening, our previously modular engineered 6-finger ZFP-A and ZFP-B were cloned into the retroviral expression vectors pMX-VP64 and pMX-SKD, while the 3-finger ZFP was only cloned into pMX-SKD. In a preliminary screening only one 6-finger ZFP (ZFP-B-VP64) and the 3-finger ZFP (3-ZFP-SKD) were further tested to evaluate the functionality to regulated endogenous *EpCAM* expression after retroviral transduction.

First, the 3-ZFP-SKD construct was tested for downregulation of *EpCAM* mRNA and protein expression in three *EpCAM* positive cell lines. In agreement with recently published data by van der Gun *et al*. (20), the 3-ZFP-SKD did not yield in significant downregulation of EpCAM expression in any examined cell line (data not shown).

Next, the ZFP-B-VP64 construct was examined to upregulate *EpCAM* expression in three *EpCAM* negative cancer cell lines and yielded in the significant upregulation of *EpCAM* mRNA expression in MDA-MB435S breast cancer cells (Fig. 4a). Preliminary flow cytometry analysis showed furthermore a subsequent increase of EpCAM protein expression (Fig. 4b).

Effect of Zinc Finger Protein ZFP-B-VP64 on EpCAM downstream target genes

EpCAM has been shown to be involved in the maintenance of human embryonic stem cell (hESC) pluripotency and high levels of *EpCAM* expression induce an upregulation of the core pluripotency factors *OCT4*, *SOX2*, *NANOG*, *KLF4* and *KLF5 (22)*.

Therefore, we set out to investigate whether the ZFP-B-VP64 induced upregulation of EpCAM has an effect on downstream targets involved in pluripotency. Upon ZFP-B-VP64 transduction of MDA-MB435S cells mRNA levels of *NANOG, SOX2, KLF4, OCT4* and *KLF5* were analysed by quantitative RT-PCR (Fig. 5). Out of five pluripotency genes analysed only *KLF5* showed a 3.5-fold up regulation (Fig. 5b).

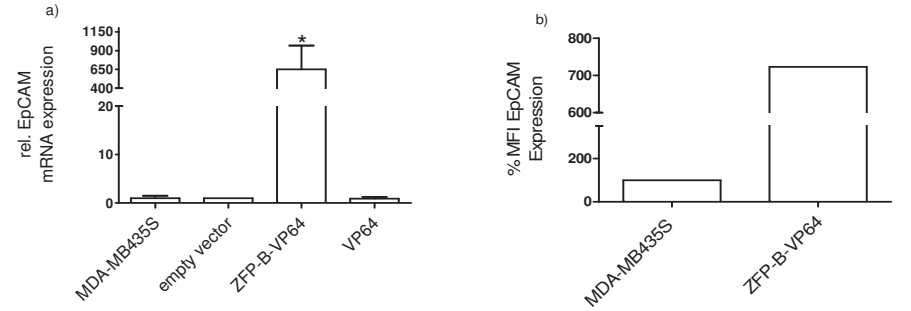

Figure 4. *EpCAM* expression in MDA-MB435S cells after retroviral delivery of the *EpCAM* targeted ZF-B-VP64. a) Upregulation of EpCAM mRNA expression by 650-fold quantified by qRT-PCR (n=3). b) Preliminary indication of EpCAM upregulation at protein level measured by flow cytometry (n=1).

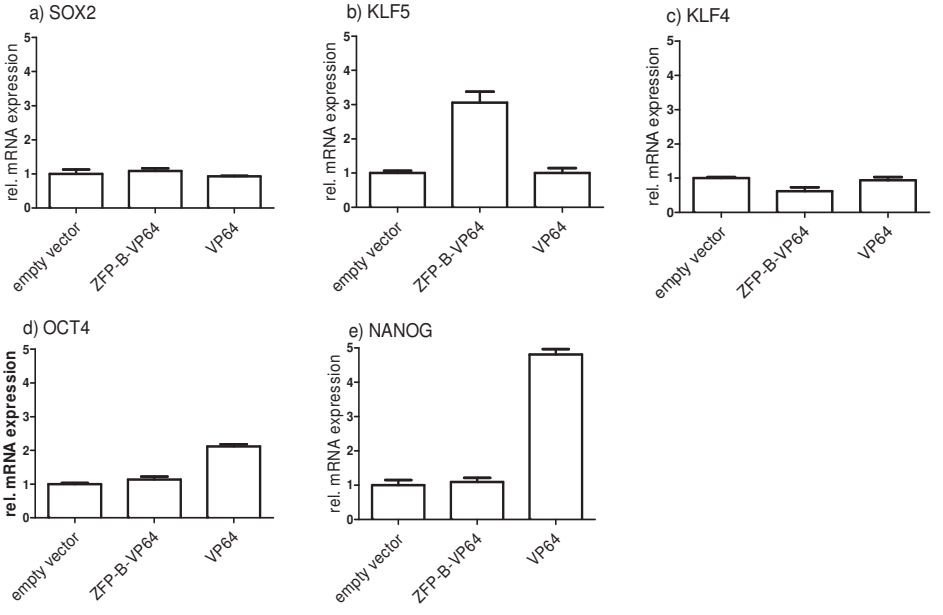

Figure 5. ZFP-B-VP64 was delivered into MDA-MB435S cells and upregulation of EpCAM expression was induced. However, artificial ZFP-B-VP64-induced upregulation of EpCAM mRNA expression does not change the expression levels of SOX2 (a), KLF5 (b), KLF4 (c), OCT4 (d) and NANOG (e) in MDA-MB435S breast cancer cells.

Effect of Zinc Finger-induced EpCAM upregulation on biological function in MDA-MB435S cells

High *EpCAM* expression is associated with increased cell proliferation and tumorigenity of breast cancer cell lines (22,23). In context of these reports we investigated whether ZFP-B-VP64 induced *EpCAM* upregulation increases the oncogenic phenotype of MDA-MB453S

cells. To this end, cell proliferation and colony formation assays were carried out upon delivery of empty vector control and ZFP-B-VP64. Neither the cell proliferation nor the colony formation assays showed any significant difference between untransduced, empty vector control and ZFP-B-VP64 transduced cells (Fig. 6 and data not shown).

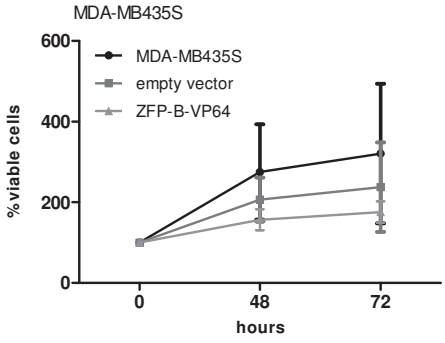

Figure 6. Cell Viability of MDA-MB 435 S cells. Up regulation of EpCAM did not change the phenotype of MDA-MB435S cells compared to Mock or empty vector transduced cells. Blue: mock-treated, green: empty vector treated, red: ATF-treated cells.

DISCUSSION

Targeted gene regulation directly on DNA level using ATFs is a promising tool to correct alternated gene expression, e.g. to re-express silenced tumor suppressor genes and to silence activated oncogenes in cancer. In this study we targeted the *EpCAM* promoter using ATFs to either up or downregulate endogenous *EpCAM* expression. Two different approaches were explored to identify ATFs which efficiently regulate *EpCAM* expression: 1) rational design of ZFPs binding in the proximal promoter of *EpCAM*, fused to transcriptional effector domains and 2) screening of a 6-finger ZF library; targeting GNN-bp triplets to identify efficient *EpCAM* regulators in a cell culture system.

Screening of the ZF library for regulators of endogenous EpCAM was based on the functional selection of ZFPs from a large ZF-repertoire. In the past, ZF library screenings have been successfully carried out for several other genes (18,19,21,24). In the present study, we selected the primary breast cancer cell line SUM149 as a target cell line, based on its *EpCAM* expression profile. SUM149 cells consist of a small population of *EpCAM* low expressing cells, indicating that *EpCAM* downregulation (upon ATF delivery) will not result in immediate cell death and sub-culturing of the cells for several weeks after cell sorting is possible to recover ZFPs potent to modulate *EpCAM* expression. In our hands the screening of the $2x10^7$ members of a ZF library did not result in the isolation of ZFPs that modulate endogenous EpCAM expression. Noteworthy, the highly complex isolation of ZFPs using a mammalian cell system has previously shown to result only in the identification of ZFPs regulating two out of ten intended targeted genes (21). Considering the complexity of endogenous

chromatin it is not surprising that another factor is crucial for the success of the ZF library screening: The accessibility of the targeted gene promoter. Nucleosome positioning and chromatin environment are non-static events and affect regulatory capabilities in different cell types (15). Therefore, isolated ZFPs for a particular gene of interest are not necessarily inter-changeable between various cell lines (25) and screenings in multiple cell lines for one gene of interest are required.

Rational designed ATFs, consisting of a 6-finger ZFP fused to the repressive effector domain SKD were transiently delivered into EpCAM expressing ovarian cancer cell lines. Although highly transfected, no significant effect on EpCAM regulation was detected (Fig. 2). Alternatively, ZFPs were delivered into EpCAM positive and negative cancer cell lines (ovarian, breast and lung) using a retroviral expression system. Upon retroviral delivery of two rational designed ZFPs fused to the transcriptional repressor SKD and activator VP64 respectively, we successfully identified one ATF upregulating EpCAM expression in the *EpCAM* negative breast cancer cell line MDA-MB435S (Fig. 5). These findings have been followed up to further explore the functional role of *EpCAM* in ovarian cancer (20). Here we set out to investigate whether the change in EpCAM expression has an effect on the cell proliferation and anchorage independent growth in soft agar, and whether *EpCAM* upregulation leads to a differential expression of down stream target genes. The intracellular domain of EpCAM (EpICD) has been shown to bind the promoters of several pluripotency genes, e.g. *NANOG, OCT4, KLF4* and *SOX2* in human and mouse embryonic stem cells (ESC) and *EpCAM* knockdown using shRNA resulted in a downregulation of *OCT4*, *NANOG*, *SOX2* and *KLF4* (22). The authors further proposed a direct activation of gene expression of *OCT4, NANOG, SOX2* and *KLF4* by EpICD. These findings led to the question whether the expression of *OCT4, NANOG, SOX2* and *KLF4* was increased in our MDA-MB435S cells with upregulated *EpCAM* expression. However, the ATF-induced expression of EpCAM was not sufficient in MDA-MB435S cells to change the expression of the pluripotency factors tested (Fig. 5). However, thus far the regulatory effect of EpCAM on the four pluripotency genes has been shown for human and mouse ESC, and this effect might be restricted to undifferentiated ESC. Expression of these genes during stemness maintenance and differentiation respectively underlies a tight network of regulation. In later stages of differentiation, expression of these genes might be prevented on multiple levels by several layers of transcriptional and post-transcriptional mechanisms and one factor alone might not be sufficient to re-express genes critical for pluripotency. Noteworthy, the breast cancer cell line MDA-MB435S we used for transcriptional upregulation is specified as a claudin-low subtype with cancer initiating properties, ergo it harbours a more cancer-stem cell-like phenotype (26). Next to their tumor initiating capability, one characteristic for cancer stem cells is their high expression of pluripotency genes. While already highly expressing OCT4, NANOG, SOX2 and KLF4, a further upregulation of these factors above baseline expression was maybe not possible anymore. Lu et al. showed shRNA-related EpCAM downregulation, resulting in a downregulation of OCT4, NANOG, SOX2 and KLF4 (22).

In different cancer models it has been shown, that knockdown of EpCAM using siRNA decreased invasion and cell proliferation (27-30). Therefore, we asked whether in turn,

upregulation of EpCAM in initially EpCAM low expressing parental cells would lead to an increase of cell proliferation. In our studies, the upregulation of *EpCAM* mRNA and protein did not lead to an increased proliferation of MDA-MB435S breast cancer cells as shown in Fig. 6. MDA-MB435S cells have a low baseline *EpCAM* expression and it is possible that the upregulation of EpCAM protein expression achieved by ZFP-B-VP64 did not overcome a critical level of *EpCAM* expression necessary for increased cell proliferation. Our results are partly consistent with results by Gostner et al, examining proliferation of *EpCAM*-low/negative breast cancer cell lines stable transfected with *EpCAM* cDNA (31). They detected only in one of two induced *EpCAM* overexpressing cell lines a minimal increase in cell proliferation. These findings suggest a cell type dependent effect of *EpCAM*.

The present study provided the basis to a manuscript to explore the functional role of EpCAM in ovarian cancer and was recently published (20).

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Chapter 4

Targeted silencing of the oncogenic transcription factor SOX2 in breast cancer

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Nucleic Acids Res. 2012 Aug;40 (14):6725-40.

ABSTRACT

The transcription factor (TF) *SOX2* is essential for the maintenance of pluripotency and self-renewal in embryonic stem cells. In addition to its normal stem cell function, *SOX2* over-expression is associated with cancer development. The ability to selectively target this and other oncogenic TFs in cells, however, remains a significant challenge due to the 'undruggable' characteristics of these molecules. Here, we employ a zinc finger (ZF)-based artificial TF (ATF) approach to selectively suppress *SOX2* gene expression in cancer cells. We engineered four different proteins each composed of 6ZF arrays designed to bind 18 bp sites in the *SOX2* promoter and enhancer region, which controls *SOX2* methylation. The 6ZF domains were linked to the Kruppel Associated Box (SKD) repressor domain. Three engineered proteins were able to bind their endogenous target sites and effectively suppress *SOX2* expression (up to 95% repression efficiencies) in breast cancer cells. Targeted down-regulation of *SOX2* expression resulted in decreased tumor cell proliferation and colony formation in these cells. Furthermore, induced expression of an ATF in a mouse model inhibited breast cancer cell growth. Collectively, these findings demonstrate the effectiveness and therapeutic potential of engineered ATFs to mediate potent and long-lasting down-regulation of oncogenic TF expression in cancer cells.

INTRODUCTION

Transcription factors (TFs) are crucial molecules orchestrating gene programs involved in self-renewal, differentiation and organism's developmental patterning. Maintaining the proper threshold of expression of TFs is critical for the normal homeostatic function of cells and tissues. Aberrant regulation of TF expression is frequently found in human malignancies and associated with specific tumor subtypes (1). Over-expression of oncogenic TFs is well documented in the mammary gland, particularly in poorly differentiated, triple negative breast cancers (TNBCs) (2). TNBCs are characterized by the lack of expression of Estrogen Receptor (ER^-), Progesterone Receptor (PR^-) and Epidermal Growth Factor Receptor 2 ($Her2^-$). Recent progress revealed that some TNBCs belonging to the basal-like and claudin-low intrinsic subtypes of breast cancers are highly aggressive and resistant to treatment (3–5). It has been proposed that these breast cancers are enriched in stem cells, which might be critical for tumor initiation, progression and resistance to chemotherapy and radiation (6–11). Albeit their fundamental role in tumor etiology and progression, TFs are currently refractory to target-based drug discovery approaches due to their lack of small molecule binding pockets. Thus, novel strategies are required to efficiently silence the aberrant expression of oncogenic TFs in cancer cells. Ideally these novel approaches should restore and stably maintain the expression pattern of these TFs, like it is observed in normal epithelial cells.

The *SOX2* gene encodes a TF belonging to the high-mobility group (HMG) family (12). *SOX2* expression is critical for the maintenance of self-renewal in embryonic stem cells (ESCs) and neural progenitor cells (13–15). While *SOX2* is highly transcribed in self-renewal conditions, its promoter undergoes epigenetic silencing during the onset of differentiation of stem cells (16,17). In neural stem cells epigenetic modifications in two *SOX2* enhancer elements, SRR1 and SRR2, control the onset of differentiation gene programs (18). Thus, in the majority of differentiated cells, including mammary epithelial cells, the *SOX2* promoter is silenced (19). However, SOX2 has been detected in normal gastric mucosae and promoter silencing by DNA methylation has been reported in some human gastric carcinomas (20,21). In contrast to gastric cancers, *SOX2* has been found over-expressed in multiple malignancies. The *SOX2* gene was found amplified in a subset of squamous cell lung and esophageal cancers in which the amplification/upregulation of *SOX2* was associated with improved clinical outcome (22). Several publications report over-expression of *SOX2* in glioblastomas (23), non-small cell lung cancer (24,25), prostate cancer (26), hepatocellular carcinomas (27) and breast carcinomas (28), supporting a role of *SOX2* as an oncogene in these tissues. *SOX2* was found over-expressed in 28% of all invasive breast carcinomas and in 43% of basal-like TNBCs (29). These reports suggest that *SOX2* could activate important gene cascades involved in tumor initiation and progression and in the maintenance of a poorly differentiated state.

Previous studies targeting *SOX2* in breast cancer cell lines have shown that shRNA-mediated knock-down of *SOX2* resulted in cell cycle arrest by down-regulation of *Cyclin D1* (30). This arrest in the cell cycle was accompanied by an inhibition of tumor cell proliferation in xenograft models (30). Although shRNA or siRNA approaches are widely used to silence

gene expression, there are potential limitations associated with inhibitory RNA (RNAi). First, oncogenes are expressed at very high levels in the mammary tissue, and thereby these targets are difficult to knock-down completely by RNAi. Second, siRNAs have a transient effect in tumor cells due to the short half-life of the small RNAs, which limits the long-term effect of RNAi in tumor cells. We reasoned that molecules able to directly silence the promoter and DNA regulatory regions necessary for oncogenic transcription would result in potent transcriptional down-regulation of the targeted gene.

Direct alteration of endogenous gene expression at DNA level requires a sequence-specific DNA-recognition module and an effector domain, which modulates transcriptional activity. Zinc-finger (ZF)-based artificial transcription factors (ATFs) are currently the state-of-the art molecules able to bind genomic sequences with potentially single locus specificity (31,32). Because ZFs bind endogenous DNA sequences with high selectivity, they provide an opportunity to modify, edit, and sculpt the epigenetic and transcriptional state of endogenous promoters. In the past, several genes have been targeted with ZF-based ATFs for transcriptional up- and down-regulation of targeted promoters (33–36). Recently, our laboratory has reported ATFs able to reactivate the expression of the tumor-suppressor gene *MASPIN*, which is silenced by epigenetic mechanisms in metastatic tumor cells. Expression of our ATFs in breast cancer cells decreased tumor growth and metastasis *in vivo* (37,38). Likewise, ATFs have been designed to repress potential oncogenes, such as *Epithelial Cell Adhesion Molecule (EpCAM)*, *human Telomerase Reverse Transcriptase* (*hTERT*) and *ErbB2/ErbB3* (34,39–41). In this article, we investigated the capability of ATFs to down-regulate the oncogenic TF *SOX2* in breast cancer cell lines. Retroviral delivery of three out of four designed ATFs led to a potent (~95%) down-regulation of endogenous *SOX2* mRNA and protein expression in two breast cancer cell lines. This strong suppression of the endogenous *SOX2* promoter activity was accompanied by a long-term inhibition of tumor cell proliferation and anchorage-independent growth. Furthermore, one of our ATFs was able to efficiently inhibit tumor growth in a xenograft model of breast cancer. Importantly, repression of *SOX2* was still maintained in the tumors *in vivo* even 48 days post-injection of the tumor cells. Overall, our data outline the therapeutic potential of ATFs to effectively repress oncogenic TFs that are highly expressed in cancer cells.

EXPERIMENTAL PROCEDURE

Cell lines and cell culture

The packaging cell line 293 T-GagPol cells and the human breast cancer cell lines MDA-MB-231, MDA-MB-435s were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS, BenchMark, Gemini Bio Products) and 1% Penicillin streptomycin (Pen/Strep, Invitrogen, Carlsbad, CA). Culture media of MDA-MB-435s cells contained additionally 0.01 mg/ml Bovine Insulin (Invitrogen). MCF7 breast cancer cells were cultured in Minimum Essential Media (MEM) supplemented with 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids (NEAAs), 1 mM sodium pyruvate, 0.01 mg/ml Bovine Insulin, 10% FBS and 1% Pen/Strep. MCF7 and MCF-12A cells were cultured in DMEM containing 20 ng/ml Epithelial Growth Factor (EGF), 100 ng/ml cholera

toxin, 00.1 mg/ml Bovine Insulin, 500 ng/ml hydrocortisone, 5% Horse serum and 1% Pen/Strep. MDA-MB-468 breast cancer cells were cultured in L15 media supplemented with 10% FBS and 1% Pen/Strep. ZR-75-1 and BT549 cells were cultured in RPMI 1640 media supplemented with 10% FBS and 1% Pen/Strep. SUM102 and SUM149 cells were cultured in human mammary epithelial cell (HuMEC) media containing HuMEC supplemental bullet kit (Gibco/Invitrogen), bovine pituitary extract (Gibco/Invitrogen) and 1% Pen/Strep. For SUM149 cells media contained additionally 5% FBS. SUM159 breast cancer cells were cultured in Ham's F12 media containing 5 µg/ml Bovine Insulin, 1 µg/ml hydrocortisone, 10 mM Hepes, 5% FBS and 1% Pen/Strep. SK-Br-3 cells were cultured in McCoy's 5a Medium, supplemented with 10% FBS and 1% Pen-Strep. MDA-MB-453s breast cancer cells were cultured in Leibovitz's L15 Medium, supplemented with 10% FBS and 1% Pen-Strep. All cell lines were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA) maintained at 37°C and 5% CO_2.

ATF construction

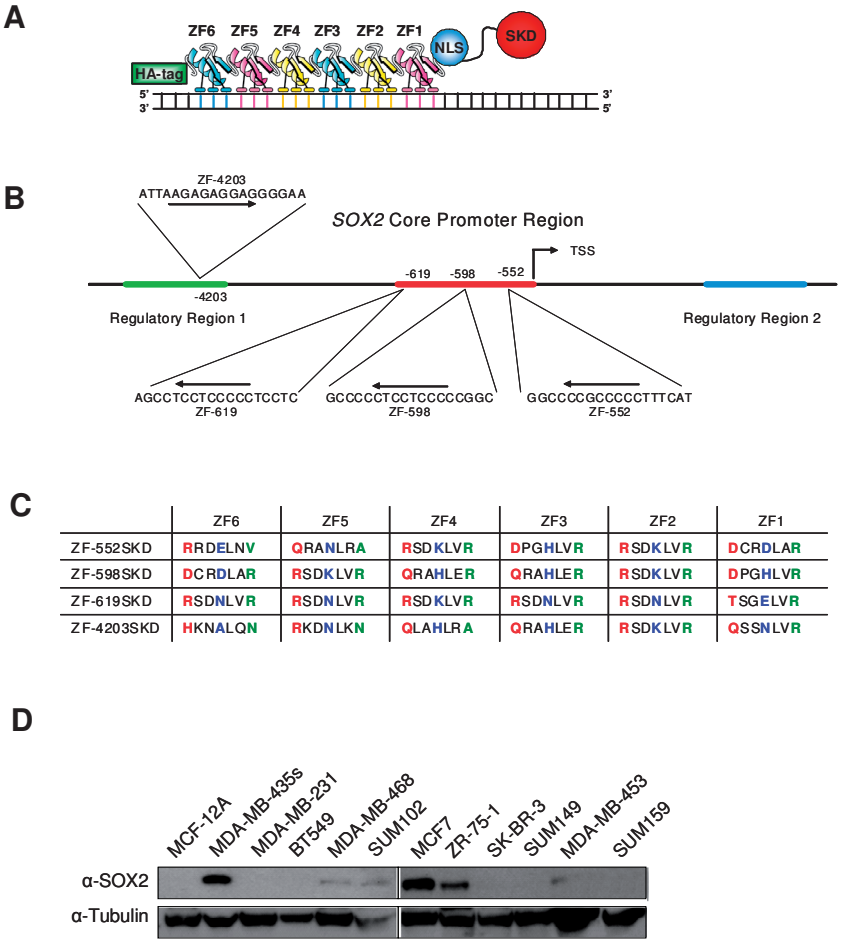
The ZF target sites within the *SOX2* promoter were selected using the website www.zincfingertools.org (42). The selection of three 18-bp target sites was based on the close proximity to the transcriptional start site and the high content of GNN-triplets in the target sequence. One ATF was designed to target an 18-bp sequence in the *SOX2* enhancer region 1, ~4-kb upstream of the TSS (Figure 1B). Specific primers were designed coding for the amino acids in the recognition helix of the ZFs responsible for the binding to the target sequence (Figure 1C). The ZF proteins were generated by overlapping PCR as described (43), *SfiI*-digested fragments were subcloned into the retroviral vector pMX-IRES-GFP-SKD, generating pMX-ZF552SKD, pMX-ZF598SKD, pMX-ZF619SKD and pMX-ZF4203SKD. Each ATF contains an internal SV40 nuclear localization signal (NLS) and a terminal hemagglutinin (HA) decapeptide tag. The correct ZF-sequence of the obtained product was confirmed by plasmid sequencing.

Retrovirus infection of MDA-MB-435s

The pMX retroviral vectors containing the *SOX2*-ATFs were first co-transfected with the plasmid (pMDG.1) expressing the vesicular stomatitis virus envelope protein into 293TGagPol cells to produce retroviral particles. Transfection was performed using Lipofectamine™ (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. For cell proliferation and soft agar assays, cells were harvested 24 h after the last infection. For flow cytometry analysis, protein, and mRNA extraction, transduced cells were harvested 48 h post-infection.

siRNA transfection

MDA-MB-435s breast cancer cells were transfected with either a *SOX2*-specific siRNA pool (siGENOME D-011778-01-04), an irrelevant (non-specific) siRNA pool targeting the TF *PATZ1* (siGENOME M-013539-00) or human a positive control for transfection, a cytotoxic siRNA pool *UBB* (siGENOME MU-013382-01-0002). The siRNAs were transfected using DharmaFECT (Dharmacon, Lafayette, CA) according to manufacturer's protocol. Cells were collected 72 h after transduction for RNA or protein preparations.



	ZF6	ZF5	ZF4	ZF3	ZF2	ZF1
ZF-552SKD	RRDELNV	QRANLRA	RSDKLVR	DPGHLVR	RSDKLVR	DCRDLAR
ZF-598SKD	DCRDLAR	RSDKLVR	QRAHLER	QRAHLER	RSDKLVR	DPGHLVR
ZF-619SKD	RSDNLVR	RSDNLVR	RSDKLVR	RSDNLVR	RSDNLVR	TSGELVR
ZF-4203SKD	HKNALQN	RKDNLKN	QLAHLRA	QRAHLER	RSDKLVR	QSSNLVR

Figure 1. Design of ATFs to down-regulate *SOX2* expression. (**A**) Schematic representation of a 6 ZF ATF bound to DNA with the orientation of the domains depicted. (**B**) Schematic illustration of the *SOX2* promoter outlining the ZF-552SKD, ZF-598SKD, ZF-619SKD and ZF-4203SKD targeted sequences and their location relative to the transcription start site (TSS). Highlighted are the core promoter (red), regulatory region 1 (green), and regulatory region 2 (blue). Arrows show the orientation of the 18-bp binding site in the promoter (from 5' to 3'). (**C**) Alpha-helical ZF amino acid sequences chosen to construct the ATFs. Residues at position –1, +3 and +6 making specific contacts with the recognition triplets are indicated in color (red refers to position –1, blue to position +3 and green to +6 of the ZF recognition helix). (**D**) Quantification of *SOX2* expression in 12 breast cancer cell lines by western blot

Generation of MCF7 stable cell lines

The coding region of the ATFs ZF-552SKD and ZF-598SKD was subcloned using the *BamHI/EcoRI* restriction sites into the expression vector pRetroX-Tight-Pur (CloneTech, Mountain View, CA). Retroviral particles from pRetroX-Tight-ZF-552SKD (ZF-552SKD), pRetroX-Tight-ZF-598SKD (ZF-598SKD), pRetroX-Tight-empty vector (empty vector), and pRetroX-Tet-On-

Advanced (pTet-On) were generated by Lipofectamin transfection (Invitrogen, Carlsbad, CA) of 293T-Gagpol cells according to manufacturer's recommendation. Virus-containing supernantant was harvested 48 h post-transfection, filtered, and concentrated using Amicon Ultra centrifugal filter Units (Millipore, Billerica, MA). MCF7 cells were co-transduced with both supernatants, one containing empty or ATF-expressing retroviral particles, and the second containing the transactivator pTet-On particles (CloneTech, Mountain View, CA) in a ratio of 1:1. Double stable MCF7 cells were selected with 2 µg/ml geneticin (Gibco/Invitrogen) and 5 µg/ml puromycin (InvivoGen, San Diego, CA) for 10 days. ATF expression was induced using Doxycycline (Dox, 100 ng/ml) for 72 h.

Lentiviral transduction of SOX2 cDNA

HEK 293T cells were transfected with a lentiviral vector encoding the *SOX2* cDNA (pSin*SOX2*, Addgene, Cambridge, MA), together with the accessory plasmids Gagpol, VSVG and RSV-REV as described (19). As control, parallel transfections were performed with empty plasmid. Viral supernatants from either *SOX2* cDNA or control transfections were used to infect the MCF7 cell lines stably transduced with either empty vector, ZF-552SKD, or ZF-598SKD, with a density of 5×10^5 cells in 10 cm plates. These cells were cultured for 48 h and cells were un-induced or induced with Dox for 72 h and then analyzed with a cell culture microscope.

Treatment with 5-aza-2′-deoxycytidine (5-Aza)

MCF7 cells stably transduced with empty vector or ZF-552SKD were plated in a density of 5×10^5 cells in 10 cm plates. Cells were un-induced or induced with Dox (+Dox) and simultaneously treated with either vehicle or 5 µM 5-Aza (+5-Aza, Sigma Aldrich, Milwaukee, WI). Cells were cultured for 48 h and then processed for quantitative real-time PCR (qRT-PCR).

qRT-PCR

Total RNA was extracted using RNeasy Kit (Qiagen; Valencia, CA) and 3 µg of RNA was converted into cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). qRT-PCR was carried out as described (38). *SOX2* mRNA expression levels were detected using Taqman primer and probes (Applied Biosystems, Foster City, CA) *SOX2* (Hs01053049s1) and *GAPDH* (FAM/MGB #4333764F). Detection of *Cyclin D1* mRNA was carried out using Absolute Blue QPCR SYBR Green Low ROX Mix (Thermo Scientific, Rockford, IL) with the following *Cyclin D1* detection primers (Applied Biosystems, Foster City, CA): forward 5′-GCTCCTGGTGAACAAGCTCAA-3′ and reverse 5′-TTGGAGAGGAAGTGTTCAATGAAA-3′. For detection of human *GAPDH* as endogenous control the primers forward 5′-CCATGTTCGTCATGGGTGTGA-3′ and reverse 5′-CATGGACTGTGGTCATGAGT-3′ were used. Data were analyzed using the comparative ΔCt method (ABPrism software, Applied Biosystems, Foster City, CA) using *GAPDH* as an internal normalization control. Data represented an average of at least three independent experiments and statistical analysis was performed using Student's *t*-test.

Nuclear extract preparation and western blotting

MDA-MB-435s cells were harvested 48 h post-transduction and MCF7 cells were harvested 72 h after Dox-induction. Nuclear protein was extracted using NE-PER Nuclear

and Cytoplasmic Extraction Reagents (Pierce, Thermo Scientific, Rockford, IL) according to manufacture's instruction. For western blot 25 µg of nuclear protein per lane was loaded and resolved on 10% pre-cast NuPAGE Bis-Tris Mini Gels (Invitrogen, Carlsbad, CA). Proteins were transferred from the gel on a Sequi-Blot PVDF membrane (BioRad, Hercules, CA). Membranes were blocked with 5% non-fat dry milk/TBST for at least 1 h, and then probed with the following antibodies: rabbit anti-*SOX2* polyclonal antibody (Cell Signalling Technology, Danvers, MA) diluted 1:1000, monoclonal mouse anti-HA-tag 1 µg/ml (Covance, Princeton, NJ) 1:2500 or anti-H3 (Active Motif, Carlsbad, CA) diluted 1:10,000. The horseradish peroxidase-conjugated secondary mouse anti-rabbit and rabbit anti-mouse antibodies were used for detection (Jackson Immunoresearch, West Grove, PA) diluted 1:10 000 and visualized using ECL plus kit (Amersham, Piscataway, NJ).

Immunofluorescence and Immunohistochemistry

MCF7 cells were plated in 24-well plates coated with fibronectin (Sigma-Aldrich, St Louis, MO). Immunofluorescence was performed using an anti-*SOX2* antibody (AB 5603, Millipore, Billerica, MA) diluted 1:200 and an anti-HA antibody 1:500 (Covance, Princeton, NJ). For staining of tumor sections we used the following antibodies: anti-*SOX2* (AB 5603, Millipore, Billerica, MA) 1:500, an anti-Ki67 antibody (ab833, Abcam, Cambridge, MA) 1:100, and an anti-HA antibody (Covance, Princeton, NJ) 1:1000. SOX2 was detected using an Alexa-Fluor555 anti-rabbit IgG (Invitrogen, Carlsbad, CA) 1:1000 dilution in MCF7 cells and 1:750 on tumor sections. Detection of the HA epitope tag was performed with an Alexa-Fluor488 anti-mouse IgG (Invitrogen, Carlsbad, CA) 1:1000 dilution in MCF7 cells and 1:500 on tumor sections. Images were taken using a confocal Leica microscope at 40× magnification.

Chromatin immunoprecipitation (ChIP) assay

Doxycycline-induced and un-induced MCF7 cells were fixed, sonicated, and incubated with either an anti-HA (Covance, Princeton, NJ) antibody or anti-RNA Polymerase II (8GW16; Covance, Princeton, NJ) antibody, respectively (1 µg/reaction). DNA complexes were immunoprecipitated using Protein A Sepharose 4 Fast Flow beads (GE Healthcare, Pittsburgh, PA). DNA was amplified by PCR using the *SOX2*-specific primers: 5'-AGTGGAAATTTTTGTTGGAG-3' and 5'-ATATACTTATCCTTCTTCATAA-3', with the following conditions: cycle 1, 5 min at 95°C; cycle 2, 1 min at 95°C; cycle 3, 1.30 min at 53°C; cycle 4, 1 min at 72°C; repeat cycle 2 to 4, 35 times followed by a final step of 10 min at 72°C. PCR-products were visualized on a 1.8% agarose gel.

Cell proliferation assays

Six replicates of MDA-MB-435s/MCF7 cells were plated in 96-well flat bottom plates in a density of 1000 cells per well. Cell proliferation was assessed every 24 h using a CellTiter Glo assay (Promega; Madison, WI) according to the manufacturer's instructions. Emitted luminescence was detected in a PHERAstar plate reader (BMG LABTECH, Durham, NC) and analyzed using PHERAstar software. Results were normalized to readings obtained immediately after seeding of the cells (day = 0). Statistical analyses were performed by 2-way analysis of variance (ANOVA).

Anchorage independent colony formation assays

For colony formation assays, 1.8% Agarose/PBS was diluted with cell culture media to a final concentration of 0.6%, and 2 ml/well media/agar solution was plated in the bottom layer of a 6 well plate. For the top layer 5000 cells were re-suspended in 0.3% media/agar solution and plated in a volume of 2 ml/well on the solidified bottom layer. The soft agar was covered with 0.5 ml culture media and cultured in 5% CO_2 humidified incubator at 37°C for 50 days. Experiments were performed in three replicates. Plates were counted visually for the presence of colonies that were greater than 2 mm in diameter. Statistical analysis was performed with a Student's *t*-test with level of significance $P < 0.05$.

Subcutaneous injections

Female NUDE mice (age 4 weeks) were purchased from Taconic Farms and housed under pathogen-free conditions. The Institutional Animal Care and Use Committee (IACUC) at the University of North Carolina at Chapel Hill approved all experiments described herein. Estrogen pellets containing 2 mg 17β-Estradiol (Sigma-Aldrich Corp. St. Louis, MO) and 8 mg Cellulose (Sigma-Aldrich Corp. St. Louis, MO), were subcutaneously implanted in the animals 7 days prior of the injection of the cells. MCF7 cells (2×10^6) were collected and re-suspended with matrigel (BD Bioscience, San Diego, CA) 1:1 volume ratio in a total volume of 100 µl. The cell–matrigel mixture was injected into the mouse flank. Tumor growth was monitored by caliper twice a week. When the tumor reached a size of approximately 50–100 mm^3, Doxycycline (+Dox) was administered to the mice in the form of green food pellets (200 mg/kg of mice chow) for a period of 28 days. During the entire experiment the mice weight was monitored to ensure absence of toxicity. Animals were euthanized 28 days after Dox induction. Statistical differences between control and ATF animals were assessed by Wilcoxon Ranks Sum Test analysis.

RESULTS

Delivery of SOX2-specific ATFs in breast cancer cells suppresses SOX2 expression

In order to down-regulate *SOX2* expression in tumor cells, we designed ATFs consisting of arrays of 6ZF domains linked to a potent repressor domain, the Kruppel Associated Box (SKD) domain. Each ZF domain recognizes 3 bp of genomic DNA, and arrays of 6ZF domains will read an 18-bp stretch of endogenous DNA (Figure 1A). Using the helix grafting or modular approach, initially developed by the Barbas group (44), we engineered four distinct ATFs. Three ATFs were designed to bind the proximal *SOX2* promoter (ZF-552SKD, ZF-598SKD, and ZF-619SKD) and one ATF (ZF-4203SKD) was directed against the *SOX2* regulatory region I (SRR1), which controls *SOX2* silencing in stem cells (Figure 1B) (18). ZF-552SKD was engineered to recognize a sequence that was perfectly conserved between the murine and the human promoters. The ZF proteins were constructed by PCR using the helix grafting approach as we have previously described (43). The specific α-helical sequences used for the assembly of the proteins are shown in Figure 1C.

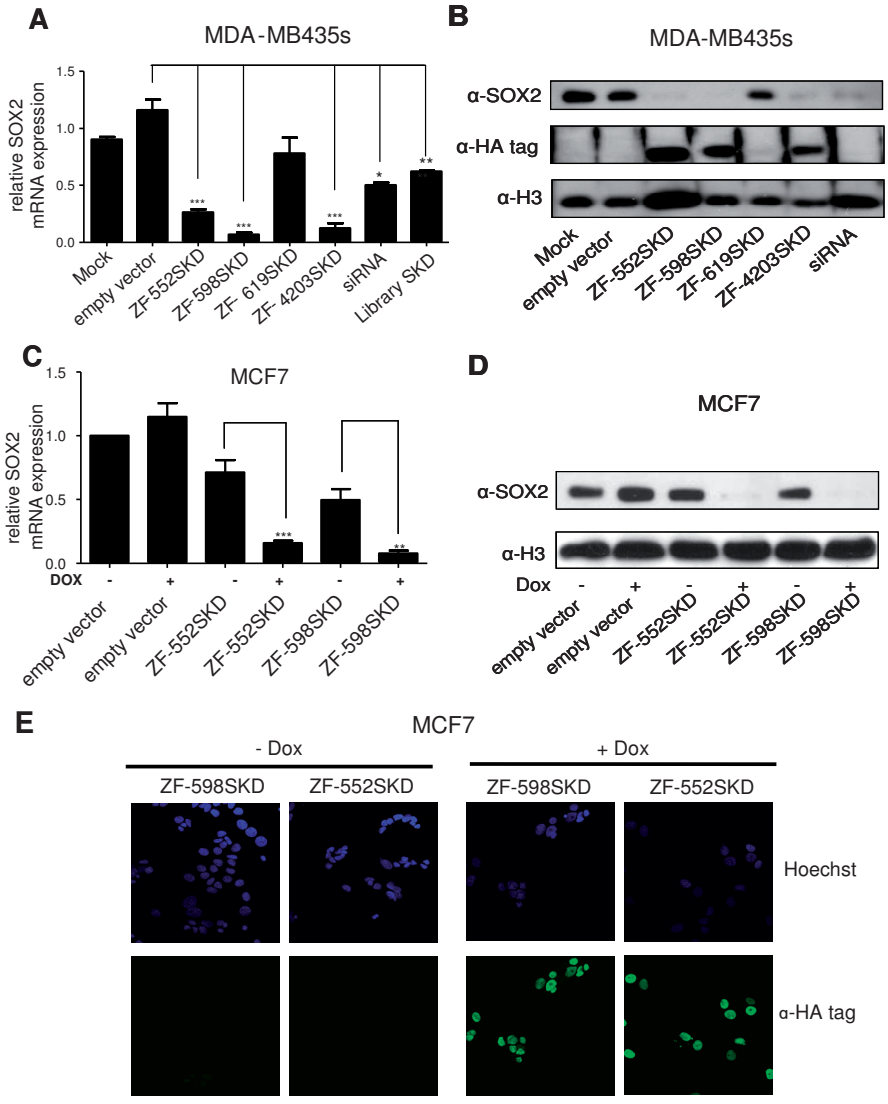


Figure 2. ATFs down-regulate *SOX2* expression in MDA-MB-435s and MCF7 breast cancer cells. (**A**) Quantification of *SOX2* mRNA expression by qRT-PCR in MDA-MB-435s cells. PMX-IRES-GFP (empty vector), ZF proteins –552SKD, –598SKD, –619SKD, –4203SKD, or a pool of 10^7 ZF domains [Library-SKD (48)] were retrovirally delivered in the cells and total mRNA was extracted. Mock-treated cells (Mock) are also indicated as control. Real-time quantification of gene expression was normalized to empty vector control samples. As positive controls for knock-down, cells were transfected with an anti-*SOX2* siRNA. A non-specific siRNA targeting another TF (*PATZ1*) was used as a negative control for siRNA transfection. Error bars represent the standard deviation of three independent experiments. Statistical significance was analyzed using *t*-test (***$P < 0.001$, *$P < 0.05$) (**B**) Detection of *SOX2* protein levels by western blot in MDA-MB-435s cells transduced with the same constructs as in (A). An anti-Histone H3 antibody was used as a loading control. ATFs were designed with a C-terminal Hemagglutinin (HA) tag, used for ATF detection. (**C**) Quantification of *SOX2* mRNA by qRT-PCR in MCF7 breast cancer cells. MCF7 cells were stably transfected with either empty vector control, ZF-552SKD, or ZF-598SKD. The ATF expression was

We first investigated SOX2 expression levels in a panel of 12 breast cancer cell lines by western blot (Figure 1D). We found that SOX2 was over-expressed in several breast cancer lines relative to non-transformed breast epithelial cells, such as MCF-12 A. The highest expression of SOX2 was detected in the ER^+ luminal MCF7 cell line, followed by the claudin-low triple negative MDA-MB-435s cell line. SOX2 was also found up-regulated in the ER^+ luminal line ZR-75-1 (Figure 1D). Gene expression microarrays have recently questioned whether the cell of origin of the MDA-MB-435 line is melanoma or basal breast cancer (45,46). More recently, with the discovery of the new mesenchymal intrinsic subtype of breast cancer (47), MDA-MB-435s cells have been clustered within the claudin-low subtype of breast cancer (5). To examine if the ATFs were able to silence the endogenous *SOX2* promoter, we chose the highest SOX2 expressing lines MCF7 and MDA-MB-435s as model cell lines.

For transduction of MDA-MB-435s cells, the retroviral vector pMX-IRES-GFP was used. These cells were transduced with up to 80–90% efficiency, as measured by flow cytometry (data not shown). Quantitative changes in *SOX2* mRNA expression upon transduction of MDA-MB-435s cells were assessed by real-time expression analyses (qRT-PCR; Figure 2A). As shown in Figure 2A, a significant down-regulation of *SOX2* mRNA expression was achieved with ZF-552SKD, ZF-598SKD but not with ZF-619SKD, relative to empty vector control. Furthermore, the targeting of the regulatory region I of *SOX2* by ZF-4203SKD led to a potent down-regulation of *SOX2* mRNA (Figure 2A). When siRNA was used to knock-down the *SOX2* mRNA, only 50% *SOX2* mRNA down-regulation was achieved by the *SOX2*-specific siRNA relative to control cells transduced with a non-specific siRNA. Importantly, ZF-552SKD, ZF-598SKD and ZF-4203SKD resulted in 74, 94 and 88% down-regulation of *SOX2* mRNA levels relative to empty vector control. Consistent with the results in Figure 2A, reduction of *SOX2* mRNA expression resulted in strong suppression of SOX2 protein expression by ZF-552SKD, ZF-598SKD and ZF-4203SKD, but not with ZF-619SKD (Figure 2B; Supplementary Figure S1). The ZF-619SKD construct was not properly expressed in the tumor cells, as assessed by western blotting using an anti-HA antibody to detect the terminal HA-tag in the ZF protein (Figure 2B). Improper translation of designed proteins could be due to instability of the protein or ineffective codon usage. Thus, this construct had no significant effect on *SOX2* mRNA expression. As an unspecific SKD control we used a diversity library of 6ZF domains comprising more than 10^7 different ZFs capable of targeting any 5′-$(GNN)_6$-3′ sequence in the genome (48). These 6ZF-library members were linked to the SKD repressor domain (library-SKD). Some down-regulation of *SOX2* (38%) was observed upon transduction of this library in the cells, which was expected based on the potential of multiple library constituents to regulate not only the SOX2 *cis*-regulatory regions but

induced by Doxycyclin as indicated in the *x*-axis (–/+ Dox). Error bars show SD of three independent experiments and statistical significance was analyzed using Student's *t*-test (***$P < 0.001$, **$P < 0.01$). (**D**) Detection of *SOX2* protein by western blot in MCF7 cells. An anti-Histone H3 antibody was used as a loading control. Samples are the same as in (C). (**E**) Immunofluorescence analysis of MCF7 cells transduced with ZF-552SKD and ZF-598SKD. Detection of ZF-552SKD and ZF-598SKD is indicated in green (α HA-tag) and nuclear staining in blue (Hoechst). The left panel shows un-induced (–Dox) and the right panel induced (+Dox) MCF7 cells. Images are taken at 40×.

also other regulatory sequences, which could indirectly affect *SOX2* expression. The effect of library members on gene expression has been well documented (48–52). However, the repressive effect of the 6ZF library was significantly lower than the effect of the proteins –552, –598 and –4203, demonstrating the sequence selectivity of the engineered ZF arrays. Similarly, the retroviral delivery of the *SOX2*-specific 6ZFs in absence of effector domain had no impact in *SOX2* transcriptional regulation. In addition, when the same 6ZFs were linked to the transcriptional activator VP64, a significant up-regulation of *SOX2* mRNA expression was achieved with ZF-598VP64 and ZF-4203VP64 in MDA-MB-435s cells (Figure 3). These data indicated that the regulatory effect of the engineered proteins required both, a sequence specific DNA binding domain and a functional effector domain. The down-regulation of SOX2 by the ZF proteins was validated at protein level by western blot (Figure 2B and D) and is quantified in Supplementary Figure S1.

We next focused on the two most potent proximal proteins, ZF-552SKD and ZF-598SKD, to assess their capability to suppress *SOX2* expression in a second cell line, MCF7. Since MCF7 cells have lower transduction efficiencies than MDA-MB-435s, we generated stable cell lines using the pRetroX-tight retroviral vector system, by which the expression of the ZF protein is controlled by Doxycyclin (Dox). The induction of ZF-552SKD and ZF-598SKD in MCF7 cells (+Dox) resulted in potent down-regulation of both *SOX2* mRNA and protein expression, compared with un-induced control (–Dox). In contrast, no change of *SOX2* expression levels were detected in +Dox cells transduced with empty vector (Figure 2C and D; Supplementary Figure S1). The expression of the ZF proteins in MDA-MB-435s and MCF7 cells was validated by western blotting and immunofluorescence (IF), respectively, using an anti-HA antibody to detect the C-terminal tag of the ZF constructs (Figure 2B and E). Collectively these results demonstrated that the ZF silencers resulted in strong suppression of SOX2 expression in MDA-MB-435s and MCF7 cells.

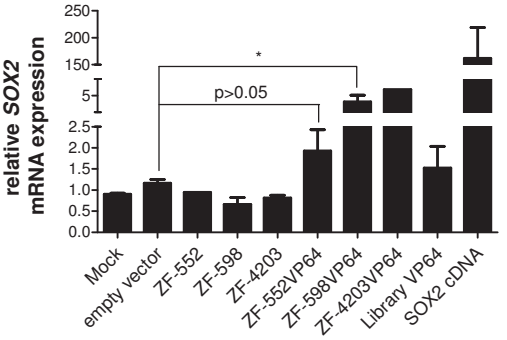


Figure 3. 6ZF domains linked to transcriptional activators enhance *SOX2* mRNA expression in MDA-MB-435s cells. Cells were retrovirally transduced with either ZF-552, ZF-598 or ZF-4203 (retroviral constructs expressing the specific DNA-binding domains but lacking the SKD effector domain or with the same ZFs linked to the VP64 transactivator domain (ZF-552VP64, ZF-598VP64, ZF-4203VP64). Library-VP64 sample refers to a pool of ~10^6 different 6ZF domains (48). Quantification of *SOX2* mRNA cells was analyzed by qRT-PCR and normalized to empty vector control. (*$P < 0.05$).

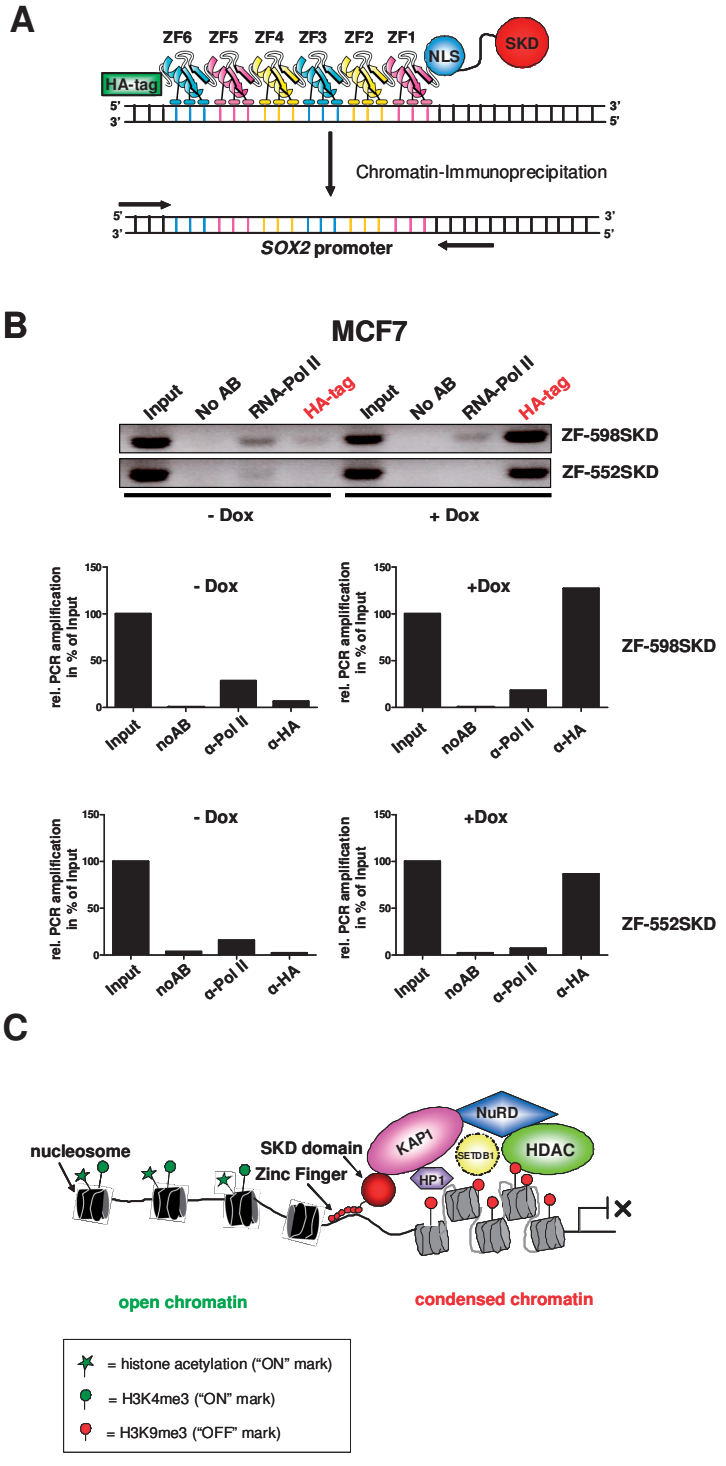
The engineered ATFs ZF-552SKD and ZF-598SKD bound their targeted DNA in vivo

To verify the binding of our engineered proteins to their target sites in the *SOX2* promoter *in vivo*, ChIP assays were performed. MCF7 cells stably transduced with either ZF-552SKD or ZF-598SKD were induced with Dox (+Dox) or maintained in –Dox media. Cells were fixed, cross-linked, and chromatin was extracted. First, ZF–DNA complexes were immunoprecipitated with an anti-HA antibody, which detects the C-terminal tag of the engineered constructs. The ChIP products were next amplified by PCR using specific primers flanking the 18-bp ZF binding sites (Figure 4A). As shown in Figure 4B induction of ZF-552SKD and ZF-598SKD by Dox led to a strong enrichment of the HA-immunoprecipitated products, indicating that the ZF constructs were binding to their target sites in the context of the endogenous *SOX2* promoter. In addition, when the ChIP experiments were performed with an anti-RNA Polymerase II (RNA Pol II) antibody, a decrease of RNA Pol II-immunoprecipitated products was detected in +Dox cells relative to the un-induced cells (Figure 4B). These experiments indicate that the engineered ZF proteins were physically associated with the *SOX2* promoter and directed potent transcriptional repression. This silencing of SOX2 expression was not induced by DNA methylation, as subsequent treatment of the ATF-transduced cells with the methyltransferase inhibitor 5-Aza-2'deoxycytidine (5-Aza) did not result in a reactivation of *SOX2* (Supplementary Figure S2). These findings are in agreement with the molecular mechanism of SKD-mediated repression (Figure 4C). In this model, SKD interacts with the co-repressor KAP1 (53). Subsequent recruitment of the nucleosome remodeling and histone deacetylase (NuRD) complex, histone deacetylases (HDACs), histone methyltrasferase (SETDB1) and heterochromatin protein 1 (HP1) catalyzes the formation of condensed chromatin, which is inaccessible for the binding of RNA Pol II.

ATF-mediated down-regulation of SOX2 expression decreased cell proliferation and anchorage-independent growth of MDA-MB-435s and MCF7 cells

Ectopic expression of the *SOX2* cDNA has been associated with an induction of oncogenic properties in different cancer cell types, including breast cancer. Reciprocally, shRNA-mediated knock-down of *SOX2* in breast (30,54) and lung (55,56) cancer cell lines resulted in decreased tumor cell growth both *in vitro* and *in vivo*. Consequently, we investigated whether the down-regulation of *SOX2* expression mediated by our *SOX2*-specific ATFs would also entail a decreased tumorigenic phenotype of breast cancer cells. To this end, MDA-MB-345s and MCF7 cells transduced with either ZF-552SKD or ZF-598SKD were first subjected to cell viability assays. We monitored cell viability of MDA-MB-435s cells transduced with either empty vector, ZF-552SKD or ZF-598SKD over time for a total period of 96 h (Figure 5A). We found that cells expressing ZF-552SKD and ZF-598SKD exhibited a significant reduction in tumor cell growth relative to un-transduced mock cells or empty vector-transduced cells (both ATFs $P < 0.001$). In MCF7 cells stably transduced with the same constructs, Dox treatment of the ZF-transduced cells resulted in decreased cell proliferation relative to controls, even at 120 h after seeding of the cells (Figure 5B).

To further validate that the down-regulation of *SOX2* expression by the ZF silencers resulted in a decreased tumorigenic phenotype, we performed colony formation assays,


A
ZF6 ZF5 ZF4 ZF3 ZF2 ZF1
NLS
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HA-tag
5'
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5'
SOX2 promoter
B
MCF7
Input
No AB
RNA-Pol II
HA-tag
Input
No AB
RNA-Pol II
HA-tag
ZF-598SKD
ZF-552SKD
- Dox
+ Dox
rel. PCR amplification in % of Input
- Dox
+Dox
ZF-598SKD
Input
noAB
α-Pol II
α-HA
ZF-552SKD
C
nucleosome
SKD domain
Zinc Finger
KAP1
NuRD
SETDB1
HDAC
HP1
open chromatin
condensed chromatin
= histone acetylation ("ON" mark)
= H3K4me3 ("ON" mark)
= H3K9me3 ("OFF" mark)

which monitor anchorage-independent growth (Figure 5C and D). MDA-MB-435s untransduced mock cells, empty vector, ZF-552SKD- and ZF-598SKD-transduced cells were seeded in soft agar and the number of colonies was quantified. While mock treated and empty vector transduced cells formed abundant foci in soft-agar, down-regulation of *SOX2* by either ZF-552SKD or ZF-598SKD abolished colony formation (Figure 5C). These results were also validated in the MCF7 cell line stably transduced with ZF-552SKD and ZF-598SKD, where induction of the ATFs by Dox effectively suppressed colony formation (Figure 5D).

The oncogenic properties of *SOX2* have been associated with activation of *Cyclin D1* promoter, by direct binding and trans-activation of the SOX2 TF. Reciprocally, down-regulation of *SOX2* was shown to arrest the proliferation of the breast cancer cells by down-regulation of *Cyclin D1* (30). We therefore analyzed *Cyclin D1* mRNA levels in MCF7 cells stably transduced with either ZF-552SKD, ZF-598SKD or controls. As shown in Supplementary Figure S3, induction of the ZF silencers resulted in a significant down-regulation of Cyclin D1 mRNA relative to control cells. Overall, these data demonstrate that our engineered proteins promote a down-regulation of tumor cell proliferation and anchorage independent growth.

To confirm that the phenotype of the ZF silencers in inhibiting tumor cell proliferation was dependent on the *SOX2* target, rescue experiments with the *SOX2* cDNA were performed (Supplementary Figure S4). MCF7 cells stably transduced with either ZF-552SKD or ZF-598SKD were challenged with either a *SOX2* cDNA-expressing lentiviral vector (pSin*SOX2*) or with an empty vector control. 48h after adding the lentiviral supernatants, the cells were either maintained in a –Dox medium or switched to a Dox-containing medium to activate the expression of the ZF proteins. As shown in Supplementary Figure S4, the delivery of *SOX2* cDNA in –Dox cells resulted in enhanced cell proliferation relative to control, consistent with the oncogenic function of *SOX2* cDNA in breast cancer. The delivery of the *SOX2* cDNA in +Dox cells rescued the cell proliferation phenotype of the ZF proteins. These functional assays demonstrate that the observed phenotype can be directly attributed to *SOX2* expression.

Down-regulation of SOX2 using ZF-598SKD inhibited tumor growth in a breast cancer xenogaft model in immunodeficient mice

To analyze the effect of the *SOX2* ZF silencers *in vivo*, we focused on ZF-598SKD since this protein mediated potent repression of breast tumor proliferation *in vitro*. We took advantage

Figure 4. ATFs bind their targeted site in the endogenous *SOX2* promoter. (**A**) Schematic illustration of the chromatin Immunoprecipitation (ChIP) assay. (**B**) ZF-598SKD (upper panel) and ZF-552SKD (lower panel) are binding their target sites, as assessed by ChIP using an anti-HA antibody. Genomic DNA bound by the corresponding ATF was amplified using *SOX2*-specific primers. An anti RNA-polymerase II (RNA-Pol II) antibody and no antibody (No AB) samples were used in the same assay, as positive and negative controls, respectively. A quantification of the ChIP assay by densitometry analyses of the bands from the same gels is outlined below. (**C**) A schematic illustration of the proposed repressive mechanism induced by ZF silencers in the *SOX2* promoter. Upon recruitment of the co-repressor KAP1 (KRAB-associated protein 1) and NuRD (nucleosome remodeling and deacetylase) by SKD in the targeted site, a repressive complex including HDACs (histone deacetylases), SETDB1 (histone methyltransferase), and HP1 (heterochromatin protein 1) is assembled. This repressive complex catalyzes the formation of condensed chromatin by de-acetylation of histones, demethylation of H3K4me3, and incorporation of H3K9me3.

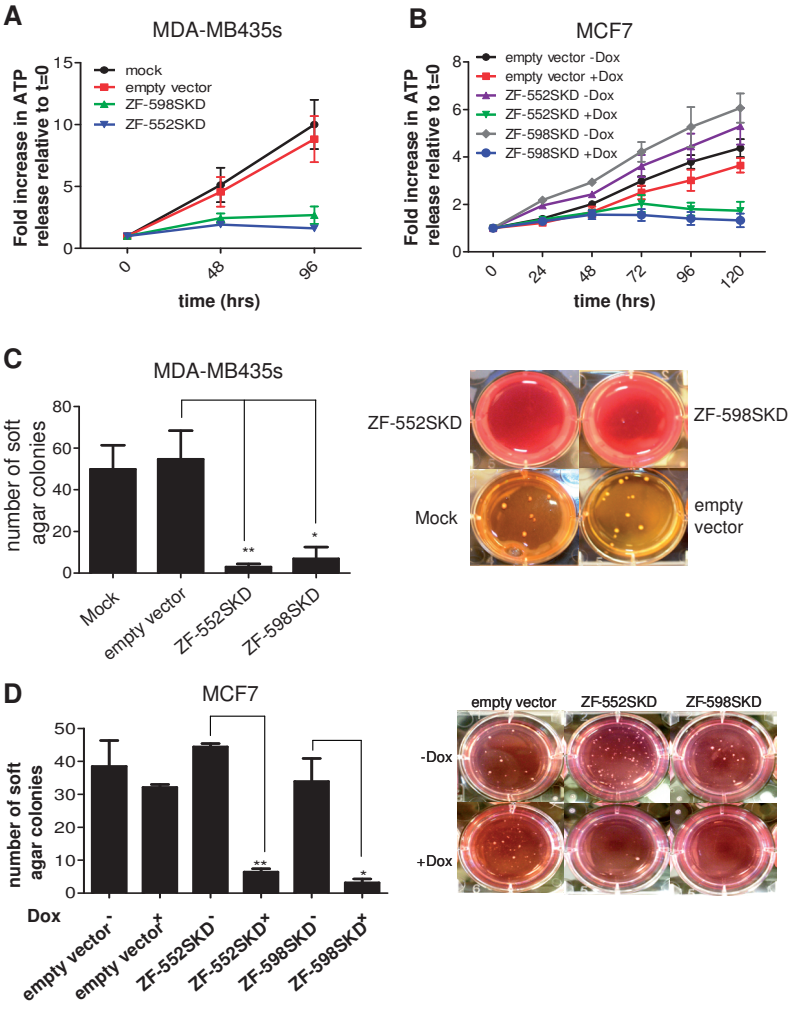

Figure 5. Repression of *SOX2* decreases cell viability and anchorage-independent growth. (**A**) Cell viability analysis of MDA-MB-435s cells transduced with either empty vector, ZF-598SKD or ZF-552SKD. Mock-transfected cells (Mock) were used to assess background. Cell viability over time was monitored over a period of 96 h after the initial seeding of the infected cells. Cell viability was monitored using a CellTiter Glo Assay (19). (**B**) Cell viability assays in MCF7 cell cells. Empty vector, ZF-552SKD- or ZF-598SKD-transduced cells were induced with Doxycyclin every 48 h. Un-induced (–Dox) cells were used as controls. The *y*-axis indicates fold increase in ATP release relative to time point 0 measured by luminescence. Statistical significance was analyzed using two-way ANOVA. The *P*-values for both, ZF-552SKD and ZF-598SKD +Dox samples versus the same samples in –Dox conditions were $P < 0.001$ at the last time point (120 h). (**C** and **D**) Anchorage-independent growth of MDA-MB-435s and MCF7 cells. (C) Quantification of the number of soft-agar colonies from un-transduced MDA-MB435s cells (Mock), MDA-MB435s cells transduced with empty vector, ZF-552SKD and ZF-598SKD. (D) Quantification of the number of soft-agar colonies from MCF7 cells transduced with either empty vector, ZF-552SKD or ZF-598SKD. –Dox and +Dox indicate un-induced and induced cells, respectively. Left panels show the quantification of colony numbers. Right panels show representative pictures of the soft-agar plates. Error bars represent SDs of three independent experiments. Statistical significance was analyzed by Student's *t*-test (**$P < 0.01$, *$P < 0.05$).

of the Tet-ON inducible ZF-598SKD and empty vector control MCF7 cell lines to analyze whether Dox induction of the ZF repressor resulted in long-term repression of *SOX2* and decreased tumor cell growth in a mouse model. Unlike constitutive viral vectors, inducible systems have the unique capability to interrogate the role of the therapeutic agent when tumors are already established (37). A total of 2×10^6 MCF7 cells stably transduced with either ZF-598SKD or empty vector control were implanted into the flank of nude mice. Tumor growth was monitored every other day using a digital caliper. Tumor volume was determined by measurement of length (L) and the width (W) as described (37). When the tumors reached ~50 mm^3 (at day 21 post-injection) half of the animals for each group ($N = 6$) were switched to a Dox-containing diet (+Dox), whereas the other half ($N = 6$) was maintained in Dox-free diet (–Dox). As shown in Figure 6A, ZF-598SKD animals induced with Dox underwent a significant inhibition of tumor growth relative to the dox-free diet (–Dox) animals. In contrast, control tumors maintained an exponential growth during the entire experiment. Moreover, the ZF-mediated inhibition of tumor growth was evident in most of the animals even 27 days post-induction (Figure 6B). A significant ($P = 0.015$) reduction of tumor growth was observed in ZF-598SKD induced animals relative to the ZF-598SKD –Dox animals. In contrast, empty vector animals did not exhibit significant reduction on tumor volume upon induction with Dox ($P = 0.269$) (Figure 6C). Examination of the tumors by qRT-PCR demonstrated that repression of *SOX2* was maintained in ZF-598SKD induced animals relative to un-induced ZF-598SKD and controls (Figure 6D). Pathological analysis of ZF-598SKD –Dox tumors by hematoxylin-eosin staining revealed an amorphous tissue with higher density of closely packed tumor cells (Figure 6E, left panel). The same morphology was found in tumors derived from empty vector control (data not shown). In contrast, the ZF-598SKD +Dox tumors exhibited a more organized tissue with increased amount of intervening stroma separating small islands of tumor cells (Figure 6E, right panel). In addition, immunofluorescence analyses of the tumor sections demonstrated that the ZF proteins were expressed in the nucleus of the majority of tumor cells in ZF-598SKD +Dox animals, but not in un-induced animals (Figure 7A) or controls (data not shown). This induction of ZF expression was accompanied by a decreased nuclear SOX2 staining (Figure 7A), and by a decreased proliferation of the tumor cells, as indicated by a Ki67 staining of the tumor sections (Figure 7B). In summary, our *in vivo* analyses indicated that the tumor suppressive functions of the engineered silencers were maintained after long-term inoculation of the tumor cells, resulting in the maintenance of the SOX2 down-regulation and decreased tumor cell proliferation in animal models of breast cancer.

DISCUSSION

In this study, we investigated the capability of ATFs to promote sequence-specific silencing of the oncogenic transcription factor (TF) *SOX2*. *SOX2* is a self-renewal TF crucial to maintain pluripotency in embryonic stem cells (ESCs) (13,14). During differentiation of ESCs, self-renewal gene promoters undergo several layers of epigenetic silencing by means of DNA, H3K4, H3K9 and H3K27 methylation (57–59). Although the function of *SOX2* in the normal mammary gland hierarchy has not been well explored, our lab has found that the gene

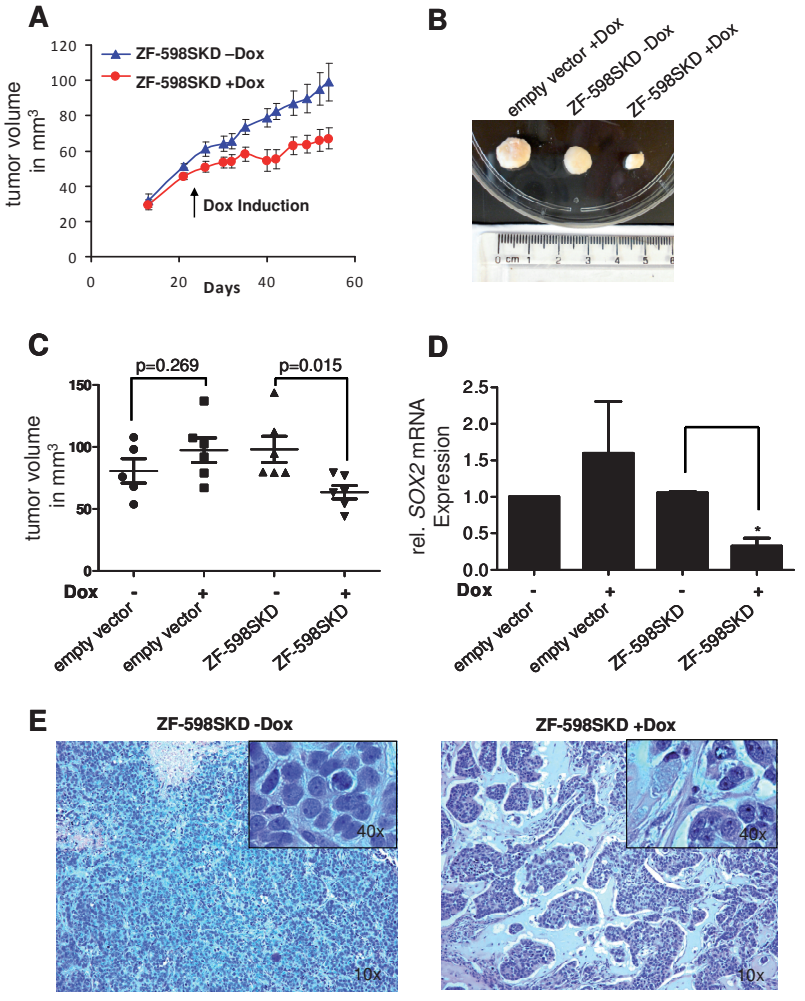

Figure 6. A *SOX2*-specific ATF inhibits the growth of pre-existing s.c xenografts of MCF7 cells. (**A**) Time course plot of tumor volume monitored by caliper measurements. Animals ($N = 6$) were either maintained in a Dox-free diet (-Dox) or induced with Dox diet (arrow) at day 21 post-injection. (**B**) Picture of representative tumors collected at day 28 post-induction from induced empty vector, un-induced ZF-598SKD, and induced ZF-598SKD animals. (**C**) Tumor volume measurements at day 21 post-induction from empty vector and ZF-598SKD groups ($N = 6$ animals per group). Differences between groups were assessed by a Wilconxon rank sum test. (**D**) Quantification of *SOX2* mRNA expression by qRT-PCR in tumor samples from a representative tumor xenograft. Bar graphs represent the mean and SD of three tumor samples. Differences in gene expression were calculated with a Student's *t*-test, $*P = 0.01$ (**E**) Hematoxylin-Eosin staining of representative ZF-598SKD –Dox and +Dox tumor sections. Un-induced (–Dox) animals revealed highly compact tumors. Induced (+Dox) ZF-598SKD sections comprised discrete islands of tumor cells, separated by intervening stroma. Pictures were taken at 10× and a detail of a 40× magnification is shown.

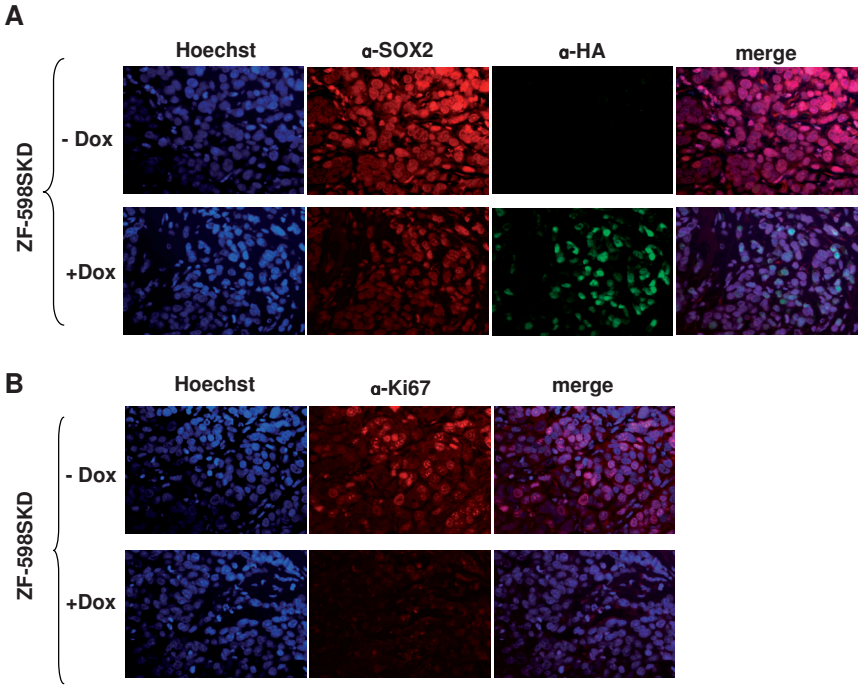

Figure 7. Induction of ZF-598 SKD reduces *SOX2* expression and tumor cell proliferation *in vivo*. (**A**) *SOX2* (red) and ZF-598SKD (α-HA, green) detection by immunofluorescence (IF) analyses of representative sections from ZF-598SKD un-induced (–Dox) and induced (+Dox) animals. (**B**) Ki67 expression (red) analyzed by IF in the same samples. Nuclei were labelled with Hoechst (blue). Images were taken at 40×.

is silenced in human mammary epithelial cells (HUMECs) derived from mammoplastic reductions (19). In contrast, over-expression of *SOX2* is frequently associated with the development of many malignancies, including breast cancer (30,54). Over-expression of *SOX2* in breast carcinomas has been associated with disease progression and poor clinical outcome (28). It has been proposed that *SOX2* is expressed in a subpopulation of cells within the tumor with tumor-initiating characteristics (2). This subpopulation of cells shares remarkable similarities in their overall gene expression profiles with stem cells and exhibit important phenotypic characteristics, such as sustained proliferation and resistance to apoptotic insults (60). Therefore, being able to target *SOX2* and other TFs involved in tumor initiation and maintenance would provide a unique opportunity for anti-cancer intervention. However, because of their lack of small molecule binding pockets, TFs are currently an example of 'undruggable targets'. Thus, novel strategies to effectively down-regulate these targets are required; these agents are anticipated to block specific gene programs involved in the maintenance of proliferation of the bulk of the tumor, stably abolishing tumor growth.

Previously, knock-down experiments using shRNAs targeting *SOX2* demonstrated that down-regulation of *SOX2* in cancer cells resulted in decreased tumor cell proliferation by down-regulation of Cyclin D1 and induction of cell cycle arrest (30,61). Although RNAi is widely

used to induce specific gene silencing, one potential limitation of interference approaches has been the achievement of complete knock-down of highly expressed gene transcripts, such as oncogenic TFs. In contrast with post-transcriptional approaches, transcriptional and epigenetic silencing of targeted genes provides additional advantage since only two genomic copies of the target promoter need to be silenced. Such genome-based approaches would prevent gene expression by silencing the promoter with no opportunity for 'residual' oncogenic transcriptional activity. In addition, genomic approaches have the unique property to impact the epigenetic state of the targeted promoter, which have the potential to enhance the longevity of the silencing and the therapeutic effect *in vivo*. Indeed, we have recently demonstrated that 6ZF proteins can target DNMT3a into specific promoter sites *in vivo*, resulting in stable, phenotypic reprogramming of the tumor cell (62).

In order to down-regulate *SOX2* expression directly at DNA level, we generated four sequence-specific ZF DNA-binding domains (31). Three ATFs were designed to bind the core promoter of *SOX2* in close proximity to the transcriptional start site (TSS) and one ATF was designed to bind in the regulatory region of SOX2 (4200 bp upstream the TSS). These multi-modular ZF genomic 'readers' were linked to the transcriptional repressor domain Kruppel-Associated box (SKD domain) (63). SKD recruits the co-repressor KRAB-associated protein 1 (KAP1). By assembling a complex with heterochromatin protein 1 (HP1), the histone methyltransferase SETDB1, nucleosome-remodeling (NuRD) and histone deacetylases (HDAC), KAP1 facilitates heterochromatin formation through methylation of H3K9 (53). In this manuscript the SKD domain was recruited to the *SOX2* promoter *via* the 6ZF proteins to promote gene silencing and chromatin condensation in breast cancer cells lines expressing high levels of *SOX2*. Our ChIP analyses demonstrated that retroviral delivery of our ZF proteins results in decreased RNA-Pol II recruitment to the *SOX2* promoter. These results support the notion that the ATFs were able to impact the epigenetic state of *SOX2* by preventing the binding of the transcription complex. The ATF-induced condensation of active chromatin is most likely not based on DNA-methylation, since co-treatment of ATF-transduced cells with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-Aza) failed to rescue *SOX2* expression (Supplementary Figure S2). Instead, the SKD domain could induce specific histone deacetylation and/or histone methylation resulting in potent *SOX2* silencing and chromatin condensation.

The importance of the epigenetic modifications in the regulation of *SOX2* and in the phenotype of tumor cells has been well documented in other reports. The *SOX2* promoter has been found hypomethylated in glioblastoma tumor specimens as compared with normal cell lines or normal adjacent tissue (64,65). Treatment of SOX2-negative glioma cell lines with 5-Aza resulted in re-activation of the endogenous gene thereby supporting the role of DNA methylation as a critical regulator of *SOX2* silencing in glioblastoma (64). In addition to DNA methylation maps, genome-wide high-throughput profiling of histone modifications of embryonic, pluripotent and lineage-committed cells demonstrated that specific histone modifications, such as H3K4me3, H3K27me3, could play a role in determining the transcriptional state of *SOX2*. In embryonic stem cells, the *SOX2* locus presented a high abundance of H3K4me3 marks, together with an enrichment of H3K36me3 in the 3' of the

gene. Moreover, *SOX2* was found flanked by two bivalent CpG islands, which could poise the gene for repression (66). In this regard, more analyses need to be performed in the breast cancer cells to uncover the role of specific histone combinations in the transcriptional status of *SOX2* and the resulting phenotypic outcomes.

Three out of four ATFs mediated strong silencing of *SOX2* mRNA expression, even with higher potency than siRNA. The ATFs ZF-552SKD and ZF-598SKD, designed to bind the core promoter region, down-regulated *SOX2* mRNA expression by 74 and 94%, respectively, and thus, nearly abolished expression of *SOX2* in MDA-MB-435s cells. ZF-4203SKD, which was designed to bind the enhancer regulatory region I (18), resulted in 88% repression of *SOX2* expression. This finding demonstrated that ATFs targeting regulatory regions in chromatin promote potent down-regulation of endogenous promoter activity. In our hands, the modular approach for engineering of ZF proteins yielded 75% success rate; hence three out of four ZF proteins were able to silence a highly expressed oncogene in breast cancer cells. When ZF-552SKD and ZF-598SKD were expressed in MCF7 cells by means of inducible retroviral vectors, an arrest in tumor cell proliferation was observed. Our xenograft experiments demonstrated that ZF-598SKD inhibited tumor growth of breast cancer cells *in vivo*, and this inhibitory phenotype was maintained long-term, even 48 days post-injection. Pathological examination of the tumors revealed that ZF-598SKD induced animals exhibited decreased proliferation, as demonstrated by Ki67 staining, relative to un-induced or control tumors. In addition, expression of the ZF proteins and stable down-regulation of *SOX2* in the tumors was validated by qRT-PCR and immunofluorescence. Interestingly, the hematoxylin-eosin staining of the ZF-598SKD induced tumors revealed small structured islands of tumor cells separated by large areas of intervening stroma, free of tumor cells. This phenotype was in contrast with the highly dense and compact growth of the tumor cells in un-induced and control tumors. The significance of this distinct phenotype induced by the ZF proteins is not known. However, it is reminiscent with the notion that transcriptional and/or epigenetic silencing of *SOX2* could induce cell arrest resulting in a more structured or normal-like growth of the tumor cells *in vivo*.

To date, multimodular proteins composed of 6ZF domains represent the state of the art molecules for the engineering of designer transcription factors since they are potentially capable of regulating single genes (32). The specificity of our 6ZF silencers for *SOX2* was further evaluated by *SOX2* cDNA rescue experiments, which suggested that the cell proliferation defect mediated by the ATFs was dependent on the down-regulation of SOX2. Nevertheless, we are currently performing the genome-wide mapping of 6ZF binding sites by ChIP-seq in our MCF7 cell lines stably expressing the 6ZFs. These experiments will provide important insights regarding the endogenous specificity of our proteins in the breast cancer genome.

Previously our group has reported the ATF-mediated re-activation of the tumor suppressor gene *Mammary Serine Protease Inhibitor* (*MASPIN)* (43). We demonstrated that *MASPIN* reactivation in breast cancer cells resulted in tumor and metastasis suppression in breast cancer and non-small cell lung carcinoma cell lines (37,38). The reactivation of *MASPIN* using the VP64 activator domain was mediated at least partially by DNA demethylation

(38). Herein we have reported the capability of the SKD domain to down-regulate highly expressed oncogenic TFs in breast cancer cells. Overall these results indicate that ATFs can modify the transcriptional landscape of tumor cells to direct cell fate. Thereby, this work opens the door to design an 'alphabet' of chromatin 'editors', with the ultimate goal to stabilize the longevity of the epigenetic, transcriptional, and phenotypic state. Ideally, such 'ZF editors' will be able to reprogram the tumor cells epigenetic landscape like it is observed in normal epithelial cells.

Metastatic resistance and disease recurrence, which ultimately affect multiple pathways, including activation of 'undruggable' oncogenic TFs, are presently the main causes of death of cancer patients. Novel treatments able to suppress disease recurrence pathways will provide great hope for targeting this disease, potentially in combination with small molecules. Moreover, the delivery *in vivo* of ATFs into the tumor cells has historically been a major challenge and limitation for clinical applications. To this aim, we are developing targeted nanoparticles encapsulating chemically modified ATF-encoded mRNA. RNA-based delivery of nanoparticles circumvents several problems associated with plasmid-based DNA delivery. RNA has a negligible chance of integration in the chromosome, it is less toxic, and less immunogenic than DNA. The *in vitro* synthesis of RNA incorporating ribonucleotide analogues enhances the stability of the RNA and the half-life inside the cells (Wang *et al.* submitted for publication). Systemic delivery of nanoparticles encapsulating an ATF-mRNA designed to up-regulate the *MASPIN* promoter in ovarian cancer cell lines demonstrated potent regulation of the endogenous gene and robust therapeutic effect *in vivo* (Lara *et al.*, submitted for publication). These data confirm that ATFs can be delivered into the tumors *in vivo* and achieve targeted and potent anti-tumor effects. In the future, encapsulation of multiple agents, for example small molecule inhibitors in combination with chemically modified RNA, which has been successfully performed with siRNA and doxorubicin in prostate cancer cells (67), could improve therapeutic outcome. Delivery of multiple agents together with ZF-encoded mRNA or even protein (68), is particularly interesting given the inherent plasticity of ZF domains to be designed for oncogenes and tumor suppressor genes, the availability of epigenetic editors, which could stabilize the longevity of the therapeutic effect *in vivo*, and the reported synergisms of ATFs with chromatin remodeling drugs (69). In summary, our data suggest that the targeted down-regulation of highly expressed oncogenes using ATF-based technologies can be used as a powerful tool for the long-term targeting of oncogenic TFs with potential application in cancer biology and other human diseases.

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Chapter 5

Epigenetic Reprogramming of Cancer Cells via Targeted DNA Methylation

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Epigenetics 2012 Apr;7(4):350-60.

ABSTRACT

An obstacle in the treatment of human diseases such as cancer is the inability to selectively and effectively target historically undruggable targets such as transcription factors. Here, we employ a novel technology using artificial transcription factors (ATFs) to epigenetically target gene expression in cancer cells. We show that site-specific DNA methylation and long-term stable repression of the tumor suppressor *Maspin* and the oncogene *SOX2* can be achieved in breast cancer cells via zinc-finger ATFs targeting DNA methyltransferase 3a (DNMT3a) to the promoters of these genes. Using this approach, we show *Maspin* and *SOX2* downregulation is more significant as compared to transient knockdown, which is also accompanied by stable phenotypic reprogramming of the cancer cell. These findings indicate that multimodular Zinc Finger Proteins linked to epigenetic editing domains can be used as novel cell resources to selectively and heritably alter gene expression patterns to stably reprogram cell fate.

INTRODUCTION

Epigenetic processes, such as DNA methylation and histone post-translational modifications, ultimately define active or inactive states of chromatin that control gene expression [1]. In general, DNA methylation is associated with repressive, inactive chromatin and is a key stable and heritable epigenetic modification [2]. The majority of 5-methylcytosine methylation occurs at CpG dinucleotides that are modified up to 70-80% in a cell type-specific manner in human cells and are faithfully transmitted to daughter cells during cell division [3, 4]. DNA methylation is catalyzed by DNA methyltransferase enzymes DNMT1, DNMT3a, and DNMT3b. DNMT1 maintains existing DNA methylation patterns, whereas DNMT3a and DNMT3b are mainly involved in the *de novo* establishment of DNA methylation marks [5, 6]. Neoplastic transformation is associated with alterations in DNA methylation, including both global hypomethylation and gene-specific hypermethylation [7]. Gains in DNA methylation in cancer cells typically reflect hypermethylation of CpG islands in gene promoter regions, which can lead to gene silencing. Methylation-dependent gene silencing is a normal mechanism for regulation of gene expression [8]. However, in cancer cells methylation-dependent epigenetic gene silencing represents a mutation-independent mechanism of inactivation of tumor suppressor genes [9].

Significant emphasis has been placed on developing novel therapeutic strategies to selectively target and change the inappropriate gene expression patterns in cells to re-direct cell fate in human disease such as cancer. However, it is recognized that any therapeutic approach must entail the ability to generate long-lasting transcriptome changes – something not readily achieved by siRNA- and/or shRNA-mediated alteration of gene expression, which is transient in nature [10, 11]. Artificial transcription factors (ATFs) provide an alternative strategy to siRNA and shRNA, in that a DNA-binding domain (DBD) can be fused directly with a transcriptional effector domain and targeted to selective promoters in cells to mediate gene expression changes [12]. ATFs contain arrays of Cys2-His2 zinc finger (ZF) domains, which specifically interact with 3 bp (triplet of recognition) of DNA [13]. Using the alphabet of recognition between the ZF domain and the DNA triplet, multimodular proteins have been engineered [14-20] that recognize specific sequences of targeted promoters and regulate expression [12, 21]. 6ZF proteins (ZFPs) that target 18 bps represent optimized, state-of-the-art designs that exhibit high specificity/affinity, and regulate single target genes with high selectivity [22, 23].

Given the advantages and potential of ATFs, we sought to develop a novel ATF-mediated approach to epigenetically regulate genes via direct targeting of DNA methylation through the catalytic domain of DNMT3a. For this study, we focused on two genes of high relevance in cancer, which control the onset of the tumorigenic state of cancer cells: the tumor suppressor *Maspin* and the oncogene *SOX2*. The levels of expression of these two genes are critically controlled by DNA methylation [24-26]. Methylation of the *SOX2* promoter irreversibly changes cell fate by promoting a switch from a proliferative state towards a differentiation state in multiple cell types, such as stem cells and cancer cells. In contrast, methylation of the *Maspin* promoter has been associated with onset of breast cancer tumorigenesis and metastasis. Herein, we show that the delivery of DNMT3a bearing ATFs that target the

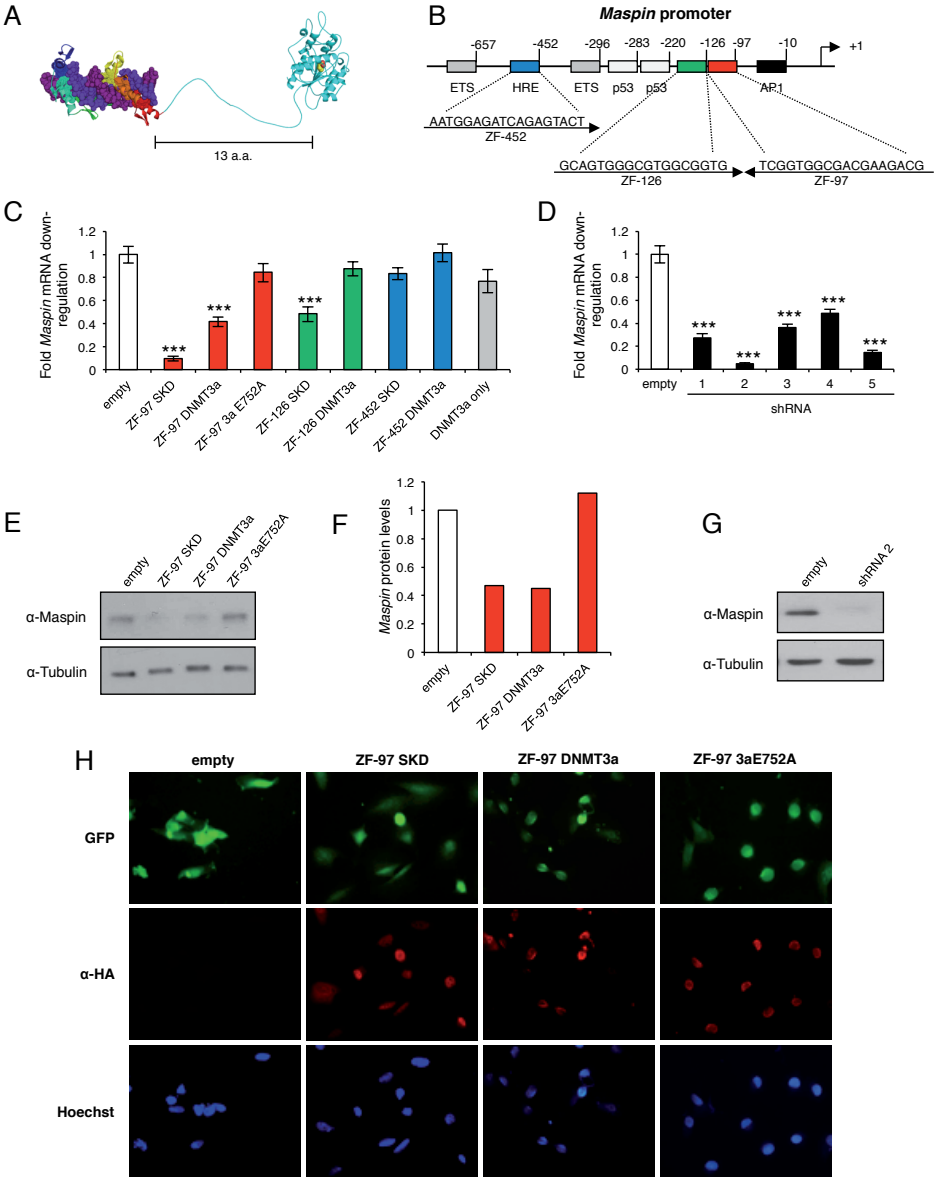

Figure 1. ATFs downregulate *Maspin* in SUM159 cells. (A) Structural model of 6 zinc finger domains linked by 13 amino acids to DNMT3a methyltransferase catalytic domain. (B) Schematic represents the ZF-452 (blue), ZF-126 (green), ZF-97 (red) sequences and their location from the transcription start site (arrow +1). (C) qRT-PCR results for *Maspin* expression in transduced cells. Red bars correspond to ZF-97 with SKD, catalytic domain of DNMT3a, and DNMT3a E752A. Green bars correspond to ZF-126 with SKD and DNMT3a domains. Blue bars correspond to ZF-452 with SKD and DNMT3a domains. Gray bar corresponds to the DNMT3a only and the white bar corresponds to empty vector control. *Maspin* expression level is depicted relative to that of the empty vector control. Error bars represent S.E.M. Statistical significance was analyzed using a t test (***P<0.0001). (D) qRT-PCR results

Maspin and *SOX2* promoters causes stable and heritable downregulation of these genes in breast cancer cells via DNA methylation. These results open the door for ATFs to be used as a therapeutic strategy for the stable regulation of gene expression patterns to control cell fate and development.

RESULTS

ATFs downregulate Maspin in breast cancer cells.

Maspin is a tumor suppressor that is silenced by DNA methylation in metastatic breast cancer cells [24, 25], contributing to metastatic progression and being associated with poor prognosis [27-30]. On the other hand, *SOX2* encodes a master regulator transcription factor (TF) and has been implicated as an oncogene in breast cancer [31, 32]. Although *SOX2* is epigenetically silenced by DNA methylation in normal epithelial cells, its promoter is overexpressed in advanced stage breast cancers, where it is essential to maintain aberrant self-renewal and tumor progression [31, 32]. Therefore, both *Maspin* and *SOX2* contribute to breast cancer initiation and development in opposite ways.

We first engineered ATFs containing ZFPs coupled with the KRAB (SKD) domain (used as a positive control) and the catalytic domain of DNMT3a to target and epigenetically repress the *Maspin* gene (**Fig. 1A**). We hypothesized that by directing the deposition of DNA methylation within the *Maspin* promoter we could trigger stable and heritable gene silencing, thereby increasing the oncogenic state of breast cancer cells. We have previously described the construction of three ZFPs designed to bind 18 bp sites in the *Maspin* proximal promoter [33] (**Fig. 1B**). The ZFPs from ZF-97, ZF-126, and ZF-452 were C-terminally coupled to DNMT3a and a catalytic mutant that abolishes its enzymatic activity (DNMT3a E752A) with a flexible linker that enables DNMT3a to methylate target sequences proximal to the ATF binding site (upstream or downstream) (**Fig. 1A**). To determine if the designed ATFs were able to downregulate *Maspin*, we expressed the ATFs using the retroviral vector pMX-IRES-GFP in SUM159 breast cancer cells that express high levels of *Maspin*. Upon ATF transduction, ZF-97 SKD, ZF-97 DNMT3a and ZF-126 SKD, but not ZF-97 DNMT3a E752A or DNMT3a domain only, significantly downregulated *Maspin* expression relative to the empty vector control (**Fig. 1C**). ZF-126 DNMT3a, ZF-452 SKD, and ZF-452 DNMT3a exhibited similar levels to that of empty vector. Therefore, ZF-97 was selected for further subsequent studies given its ability to transcriptionally repress *Maspin* with the DNMT3a catalytically active domain. Transductions with five different shRNAs that target *Maspin* demonstrated

for *Maspin* expression. Black bars correspond to five different shRNA constructs that target *Maspin* and the white bar corresponds to empty vector control. *Maspin* expression level is depicted relative to that of the empty vector control. Error bars represent S.E.M. Statistical significance was analyzed using a t test (***P<0.0001). (E) Western blot analysis of Maspin protein levels in cells transduced with empty, ZF-97 SKD, ZF-97 DNMT3a, and ZF-97 3aE752A constructs. Tubulin is used as a loading control. (F) Quantification of Maspin protein levels relative to Tubulin. (G) Western blot analysis of Maspin protein levels in cells after shRNA-mediated knockdown of *Maspin*. Tubulin is a loading control. (H) Detection of the retrovirus (GFP, green) and ZF-97 (α-HA, red) by immunofluorescence in cells. Nuclear staining was performed using Hoechst (blue). Images taken at 40x.

significant transcriptional repression compared to the empty vector control (Fig. 1D). The *Maspin* shRNA 2 stable cell line was used in the subsequent studies as a positive control. Transduction of empty and ZF-97 DNMT3a E752A had no effect on Maspin protein levels; in contrast, ZF-97 and ZF-97 DNMT3a cells exhibit significant alteration of Maspin protein levels, reflecting 50% reduction (Fig. 1E and F). The *Maspin* shRNA 2 completely abolished Maspin protein levels (Fig. 1G). Cells transduced with the empty vector, ZF-97 SKD, ZF-97 DNMT3a and ZF-97 DNMT3a E752A showed similar levels of infection based on GFP detection levels (Fig. 1H). Anti-HA was detected in all ZF-97 containing cells, but not in the empty vector (Fig. 1H). These results demonstrate that retrovirally delivered ZF-97 linked to silencing domains promoted *Maspin* downregulation in SUM159 cells similar to that of shRNA-directed silencing.

ZF-97 DNMT3a represses Maspin by targeted DNA methylation

To verify that ZF-97 DNMT3a is catalytically active and that targeted DNA methylation was dependent on the specific binding of ZF-97, SUM159 cells were transduced with empty vector, ZF-97 DNMT3a, ZF-97 DNMT3a E752A, and DNMT3a only (untargeted domain). Genomic DNA of transduced cells was bisulfite converted and sequenced, and methylation patterns were examined within the *Maspin* promoter and exon 1 (Fig. 2). ZF-97 DNMT3a directly targeted DNA methylation to the *Maspin* promoter, with the highest density of methylated CpG dinucleotides immediately downstream of the ZF-97 binding site >50% compared to controls (Fig. 2). Methylated CpG sites (50%) were found +429 downstream of the transcriptional start site (+1), which could reflect the folding and accessibility of these CpG sites within the higher order chromatin structure. Only background levels (<1%) of CpG sites were methylated in cells containing the catalytically dead mutant (E752A) and untargeted DNMT3a domain (lacking ZF-97), indicating that a functional ZFP domain and an active DNMT3a enzyme domain are required for targeted methylation *in vivo*. Cells transduced with ZF-97 SKD did not exhibit targeted DNA methylation (Suppl. Fig. 1). To determine if we could directionally target DNA methylation, we positioned the DNMT3a domain on the N-terminal region of the ZF-97 (originally cloned C-terminal). The DNMT3a ZF-97 significantly downregulated *Maspin* mRNA levels and similar levels of expression were seen in transduced cells (Suppl. Fig. 2A and B). DNA methylation was targeted to the adjacent region surrounding the ZF-97 binding site, similar to that seen with the C-terminal DNMT3a domain (Suppl. Fig. 2C). These results demonstrate the ability to selectively and efficiently target DNA methylation to the *Maspin* promoter, and that this methylation was not dependent on the orientation of the DNMT3a domain relative to ZF-97. This could be explained by the conformational freedom provided by our flexible linker separating the 6ZFs and the DNMT3a, which can mediate effective methylation in both orientations.

Downregulation of Maspin by targeted DNA methylation increases breast cancer cell colony formation

Maspin is silenced by DNA methylation in advanced breast cancers, contributing to metastatic progression. Therefore, we sought to examine if the downregulation of *Maspin* by ZF-97 DNMT3a targeted methylation led to anchorage-independent growth in soft agar

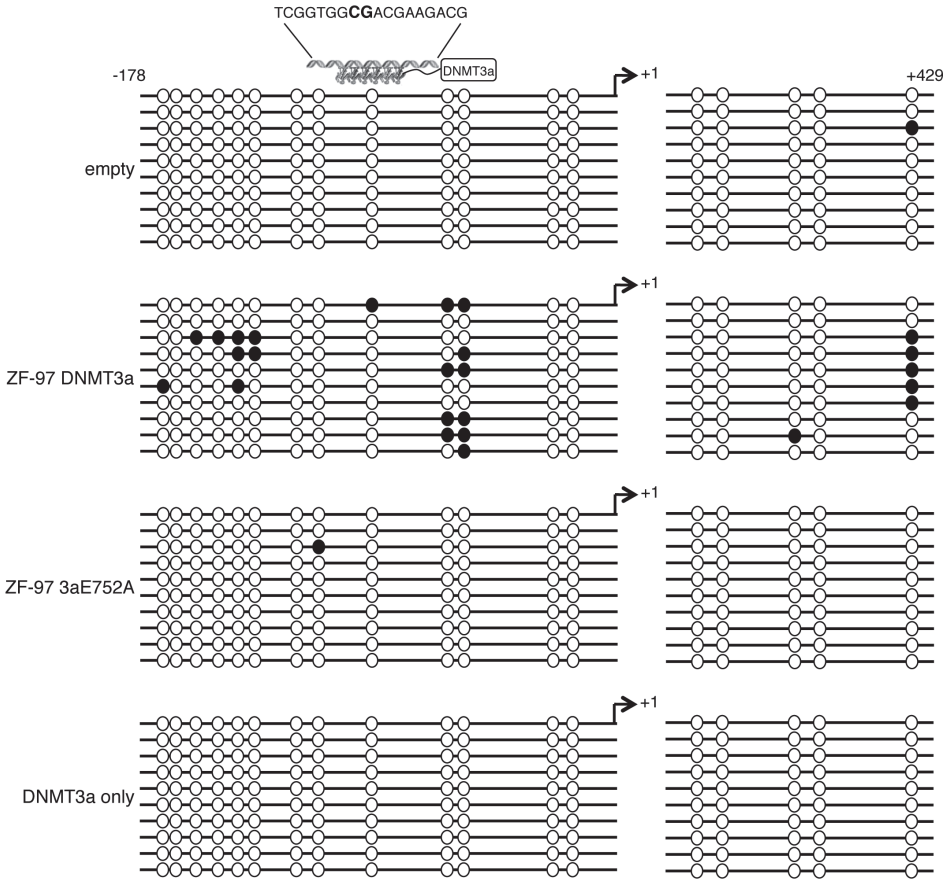

Figure 2. ZF-97 DNMT3a represses *Maspin* by targeted DNA methylation. Bisulfite sequence analysis for *Maspin*. Methylated CpGs are designated by closed circles, unmethylated CpGs are designated by open circles for cells transduced with empty vector, ZF-97 DNMT3a, ZF-97 3aE752A, and DNMT3a only (10 replicates each). Transcription start site indicated by arrow +1. ATF binding site depicted by 6 ZF proteins linked with a C-terminal DNMT3a domain with the sequence shown above.

similar to metastatic cells that lack *Maspin* expression. SUM159 is a poorly tumorigenic breast cancer cell line expressing high levels of *Maspin*. Transduced cells were seeded in soft agar and colony formation was examined. ZF-97 SKD, ZF-97 DNMT3a, and *Maspin* shRNA containing cells all induced colonies (>100 colonies) relative to empty vector, DNMT3a mutant or DNMT3a only cells (<10 colonies) (Fig. 3A and B). Select single colonies (ZF-97 DNMT3a clones 1-12) were recovered from the soft agar and expanded in culture medium. Importantly, qRT-PCR analysis of these clones revealed significant silencing of *Maspin* expression promoted by DNMT3a (Fig. 3C). Moreover, low levels of expression of the retrovirally integrated DNMT3a construct were observed via qRT-PCR compared to the parental ZF-97 DNMT3a cells (Fig. 3D). Genomic DNA from ZF-97 DNMT3a clone

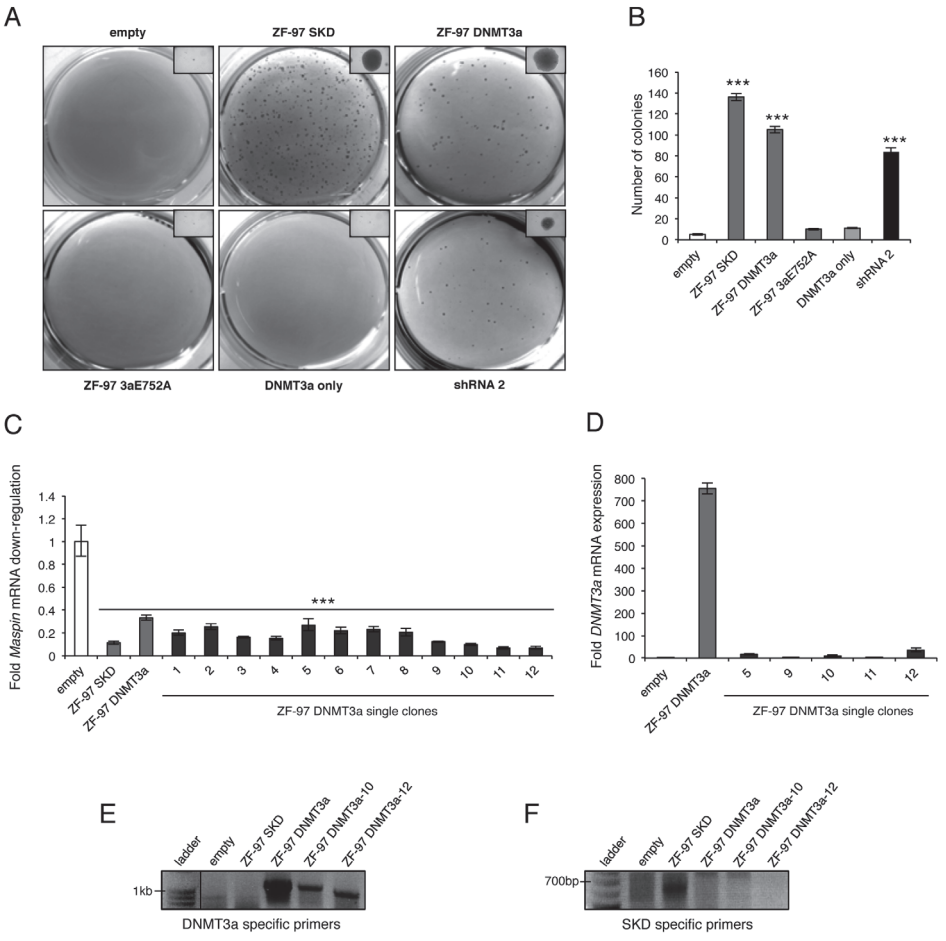

Figure 3. Downregulation of *Maspin* by targeted DNA methylation increases breast cancer cell colony formation (A) Soft agar assay results from cells transduced with empty vector, ZF-97 SKD, ZF-DNMT3a, ZF-97 3aE752A, DNMT3a only, and shRNA 2. Representative images of the entire agar well stained with Crystal Violet are shown along with a closer image of a single colony (in upper right corner). (B) Quantification of colony number. The white bar corresponds to empty vector control, the dark grey bars correspond to ZF-97, the light gray bar corresponds to DNMT3a only, and the black bar corresponds to shRNA 2. Error bars represent S.E.M. Statistical significance was analyzed using a t test (***P<0.0001). (C) qRT-PCR results for *Maspin* expression in ZF-97 DNMT3a single clones (dark red bars 1-12) picked from soft agar and grown in culture for >50 generations. The white bar corresponds to empty vector control and the grey bars correspond to ZF-97 SKD and ZF-97 DNMT3a (parental pool) included for technical reference. *Maspin* expression level is depicted relative to that of the empty vector control. Error bars represent S.E.M. Statistical significance was analyzed using a t test (***P<0.0001). (D) qRT-PCR results for the DNMT3a construct expression in select ZF-97 DNMT3a single clones (dark grey bars). DNMT3a expression level is depicted relative to that of the empty vector control and the parental ZF-97 DNMT3a is included for technical reference. (E) Agarose gels from genomic DNA extracted from cells transduced with the empty vector, ZF-97 SKD, ZF-97 DNMT3a, ZF-97 DNMT3a single clones 10 and 12. Line indicates a lane on the same gel but was separated from subsequent lanes. Amplicons demonstrate products obtained with DNMT3a specific primers and (F) SKD specific primers.

10 and 12 were examined for the presence of the retrovirally integrated DNMT3a or SKD construct, using primers designed to overlap the retroviral vector with the DNMT3a or SKD domain, respectively. The integrated DNMT3a construct was detected in all of the constructs containing the DNMT3a domain, but not in cells containing the empty vector or SKD domains (Fig. 3E). The integrated SKD was only detected in the ZF-97 SKD containing cells (Fig. 3F). These results suggest that by 50 days post-infection, the ATF retrovirus exhibits diminished expression. Yet, the cells stably maintained *Maspin* downregulation, consistent with ATF-mediated epigenetic reprogramming of the *Maspin* promoter.

Maspin is stably and heritably repressed by DNA methylation

To address whether the initial methylation pattern of the targeted gene was inherited over multiple cell generations, we first treated ZF-97 DNMT3a clones 10 and 12 with 5-aza, an inhibitor of DNMTs, and *Maspin* gene expression was restored (100% of control) (Fig. 4A). The stable downregulation of *Maspin* seen in ZF-97 DNMT3a clone 12 was due to DNA methylation patterns similar to what was observed in the parental cells (ZF-97 DNMT3a)(Fig. 4B). These data suggest that DNA methylation patterns imposed by the ZF-97 DNMT3a construct were stably and heritably maintained over multiple cell generations.

The protein UHRF1 (ubiquitin-like, containing PHD and RING finger domains 1) is necessary for maintaining DNA methylation patterns during replication by recruiting DNMT1 [34, 35]. To further verify the stable transmission of DNA methylation initiated by the ZF-97 DNMT3a ATF, we challenged ZF-97 DNMT3a clone 12 cells with siRNAs targeting the UHRF1 protein. We found that knock-down of UHRF1 (>90%) (Fig. 4C and D) led to a significant re-expression of *Maspin* relative to that of the non-targeted control (Fig. 4E). This restored *Maspin* levels to those obtained after treatment with 5-aza (Fig. 4A). Collectively, these results demonstrate that the ZF-97 DNMT3a ATF directly targets CpG methylation adjacent to the ATF binding site. Over time the ATF expression is no longer detected and required for silencing, and these sites of methylation were read and transmitted by UHRF1/DNMT1 to stably convey the methylation pattern over multiple cell generations.

ZF-552 DNMT3a downregulates SOX2 in MCF7 cells resulting in reduced cell proliferation

Some breast cancers overexpress the *SOX2* gene, and in these cancers *SOX2* behaves like an oncogene [36]. Therefore, the *SOX2* gene was targeted to determine if directed, stable silencing by DNA methylation (similar to what was observed with *Maspin*) would repress an overexpressed oncogene found in many breast cancers and, in contrast to repression of *Maspin*, if this repression could result in inhibition of cancer cell growth. A ZFP (ZF-552) was utilized coupled to the DNMT3a domain designed to bind a unique 18 bp region in close proximity to the transcription start site within the *SOX2* promoter. Upon ZF-552 DNMT3a induction (+DOX) in MCF7 breast cancer cells that overexpress *SOX2*, we observed a significant down-regulation of *SOX2* mRNA (≥60%) and protein levels (>80%) compared to the empty vector and no DOX induction (Fig. 4F and G). A time-course cell viability assay was utilized to assess changes in cell proliferation after induction of the ATF (+DOX) and ATF shut-off (DOX removal). *SOX2* repression was associated with arrest of cell proliferation

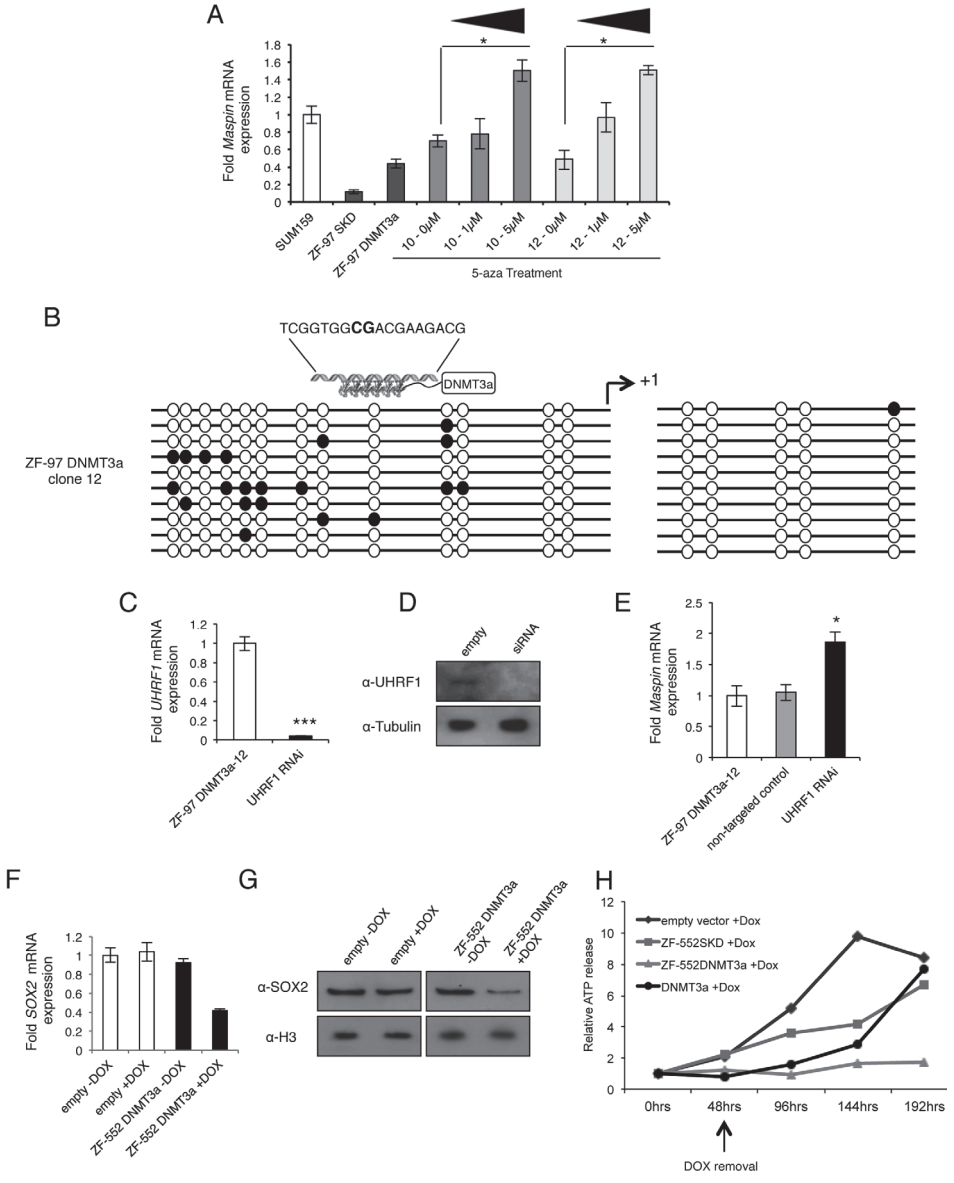

Figure 4. *Maspin* is stably and heritably repressed by DNA methylation and ZF-552 DNMT3a downregulates *SOX2* in MCF7 cells resulting in reduced cell proliferation. (A) qRT-PCR results for *Maspin* expression in ZF-97 DNMT3a single colony cells treated with 1μM and 5μM of 5-aza. Error bars represent S.E.M. Statistical significance was analyzed using a t test (*P<0.05). (B) Bisulfite sequence analysis for *Maspin* in ZF-97 DNMT3a clone 12. Methylated CpGs are designated by closed circles; unmethylated CpGs are designated by open circles (10 replicates each). Transcription start site indicated by arrow +1. ATF depicted by 6 ZF proteins linked with a DNMT3a domain with the sequence shown above. (C) qRT-PCR results for *UHRF1* expression after RNAi-mediated *UHRF1* knockdown in ZF-97 DNMT3a clone 12 cells. Black bar correspond to RNAi and the white bar corresponds to ZF-97 DNMT3a clone 12 before knockdown. *UHRF1* expression level is depicted relative to that of ZF-97 DNMT3a clone 12. Error bars

following ATF induction. However, ZF-552 DNMT3a cells maintained robust cell arrest for at least 192 hrs after DOX removal (**Fig. 4H**). In contrast, cells containing the empty vector, ZF-552 SKD or untargeted DNMT3a were able to recover and resume proliferation (**Fig. 4H**). Our results indicate that the 6ZF-DNMT3a fusions nucleated a heritable transmission of DNA methylation over cell generations that were sufficient to promote stable cancer cell growth inhibition, even when the expression of the ZF agent was suppressed.

DISCUSSION

We have generated novel molecular resources for ATF-mediated selective and heritable epigenetic repression of critical genes in cancer cells, showing for the first time that epigenetic targeting can stably reprogram cancer cells. Effector domains, such as the naturally occurring transcription factor KRAB domain (SKD), do not possess intrinsic enzymatic activity. Instead, these domains are used to repress gene expression by passively recruiting chromatin remodelers, histone-modifying enzymes, and other proteins [37]. Here, we employed DNMT3a to repress the *Maspin* and *SOX2* genes. In contrast to the action of the KRAB domain, which recruits enzymes to repress gene transcription [37], epigenetic effector domains such as the DNMT3a are active enzymes that will directly repress promoter activity. Gene repression by DNA methylation occurs via recruitment of co-repressors to methylated CpG dinucleotides, such as mSin3 or Mi2-NuRD, and histone deacetylases, to form condensed, repressive chromatin leading to stable and heritable gene inactivation [38]. The C-terminal catalytic domain of DNMT3a is active and, when fused with a ZFP, directs DNA methylation at the target sequence of reporter systems *in vitro* [39]. While repression can be produced with the use of siRNAs, these methods are of limited utility [10, 11]. For example, several regions of mRNA are generally targeted by multiple siRNAs to ensure successful targeted repression, increasing chances of off-target effects. In contrast, ATFs allow the efficient downregulation of gene expression through targeting of a single sequence. Thus, the ability to stably and heritably alter gene expression states via epigenetic reprogramming in cancer cells could be utilized for new therapeutic approaches or to target genes of specific biological significance.

The tumor suppressor gene *Maspin* was utilized as a proof-of-principle because it allowed (a) examination of the resulting tumorigenic phenotype after downregulation,

represent S.E.M. Statistical significance was analyzed using a t test (***P<0.0001). (D) Western blot analysis of UHRF1 protein levels in cells after siRNA-mediated knockdown of UHRF1. Tubulin is used as a loading control. (E) qRT-PCR results for *Maspin* expression after RNAi-mediated UHRF1 knockdown in ZF-97 DNMT3a clone 12 cells. Black bar corresponds to RNAi, the gray bar corresponds to a non-targeted control, and the white bar corresponds to ZF-97 DNMT3a clone 12 before knockdown. *Maspin* expression level is depicted relative to that of ZF-97 DNMT3a clone 12. Error bars represent S.E.M. Statistical significance was analyzed using a t test (*P<0.05). (F) qRT-PCR results for *SOX2* expression in transduced cells. White bars correspond to empty vector (- or +DOX) and black bars correspond to ZF-552 DNMT3a. (G) Western blot analysis of SOX2 protein levels in cells either un-induced (-DOX) or induced (+DOX) with empty vector and ZF-552 DNMT3a. Histone H3 is used as a loading control. (H) A representative time course cell viability assay with cells induced with DOX at t=0. At 48 hrs post-induction the DOX was removed from the cell medium. Cell viability is measured by fold increase in ATP release related to t=0. Error bars represent S.E.M.

(b) determination of the ability to repress *Maspin* by DNA methylation near the ZF-97 binding site, and (c) characterization of stable and heritable methylation patterns over multiple cell generations. There are four lines of evidence that support the suggestion that heritable DNA methylation patterns are established through the action of ZF-97 DNMT3a. First, single soft agar colonies that proliferated through ≥50 cell generations were examined and found to lack expression of the retrovirus (ZF-97 DNMT3a construct), yet *Maspin* repression was maintained. Transcriptional silencing of retroviral constructs has been previously described after long-term culture of infected cells [40, 41]. Second, select cell clones were treated with 5-aza, an inhibitor of DNMTs, and *Maspin* expression was restored to wild-type SUM159 levels. Third, DNA methylation was observed in ZF-97 DNMT3a clone 12 demonstrating the maintenance after multiple cell divisions of the original methylation pattern imposed by the ZF-97 DNMT3a. In the replication fork during cell division UHRF1 recognizes hemi-methylated DNA and interacts with DNMTs to faithfully methylate the daughter strands properly. Fourth, when UHRF1 was knocked-down in ZF-97 DNMT3a clone 12 cells, the level of *Maspin* increased similar to that observed with 5-aza treatment. This demonstrates that UHRF1 maintains the methylation pattern observed in these cells. The ability to silence the oncogene *SOX2* and to mitotically arrest cell growth by targeted DNA methylation, most likely in G0/G1, is an exciting finding given that our technology sustained permanent, indefinite silencing of *Maspin*. Whereas, the conical SKD repressor domain coupled with ZF-552 returned to levels similar to that of empty vector, ZF-552 DNMT3a cells showed arrested growth for 8 days strongly suggesting that the silencing of *SOX2* is persistent due to DNA methylation.

Delivery of ZFPs into a cell *in vivo* has been a major limitation that has provided challenges for the clinical application of ZF technologies. We and colleagues are designing novel state-of-the-art systemic delivery technologies for preclinical applications of ZFPs in mouse cancer models. We are utilizing nanoparticle technology to encapsulate ATF mRNA, which is less toxic and immunogenic than current delivery approaches. mRNA has a negligible chance of integration in the genome, therefore delivery of mRNA circumvents the problems associated with gene therapy. Overall our data demonstrate that it is possible to target "at will" DNA methylation to epigenetically and phenotypically reprogram cells. The ability to stably and heritably alter gene expression states via epigenetic reprogramming holds far reaching implications for treating human diseases, such as cancer.

MATERIALS AND METHODS

ATF Construction

The *Maspin* and *SOX2* 6ZFP construction conditions were previously defined [33]. *Maspin* ZFP sequences are shown in Figure 1B and the *SOX2* ZF-552 sequence is as follows 3'GGCCCCGCCCCCTTTCAT-5'. The construction of the KRAB domain (SKD) was previously defined [21, 42]. The DNMT3a catalytic domain (598-908 amino acids) and flanking sequences were amplified from human fibroblast DNA and cloned in frame into pMX-IRES-GFP [43]. Catalytically dead versions of DNMT3a were generated by PCR-mediated mutagenesis of the active site ENV motif, by changing ENV to ANV (E752A) [39]. The linker region between the ZFP and DNMT3a sequence is as follows: QASPKKKRKVGRA.

Cell Culture, RNA, and DNA Preparation

Human breast cancer cell lines SUM159 and MCF7 were obtained from the Tissue Culture Facility of the UNC Lineberger Comprehensive Cancer Center (Chapel Hill, NC) and the 293TGagPol cell line was obtained from the American Type Culture Collection (ATCC). Cell lines were propagated in growth medium specified by the ATCC. Cell cultures were harvested for RNA and DNA preparation after retroviral or lentiviral transduction, siRNA transfection, and following demethylating treatment using the RNeasy Kit (Qiagen; Valencia, CA) or the Puregene DNA Purification Kit (Gentra Systems; Minneapolis, PA) respectively, according to the manufacturer's protocol.

Retroviral and Lentiviral shRNA Infection

The retroviral vector pMX-IRES-GFP containing the *Maspin* ATFs and pRetroX-Tight-Pur containing the *SOX2* ATF, and pRetroX-Tet-On-Advanced was first co-transfected with a plasmid (pMDG.1) expressing the vesicular stomatitis virus envelope protein into 293TGagPol cells to produce retroviral particles. Transfection was performed using Lipofectamine (Invitrogen; Carlsbad, CA) as recommended by the manufacturer. Recombinant lentiviruses were generated using a three-plasmid system as recommended by the manufacturer (Open Biosystems; Huntsville, AL). The viral supernatant was used to infect the host cell lines (SUM159 and MCF7). pMX-IRES-GFP cells were collected 48 hrs after transduction for RNA, DNA, or protein preparation. pRetroX-Tight containing MCF7 cells were selected using 2 mg/ml geneticin (Gibco, Invitrogen; Carlsbad, CA) and 5 µg/ml puromycin (InvivoGen; San Diego, CA). Cells were induced with 100 ug/ml of doxycycline (DOX).

siRNA Transfections

SUM159 ZF-97 DNMT3a-clone 12 breast cancer cells were transfected with Human *UHRF1* (siGENOME MU-006977-01-0002) and Human *UBB* (siGENOME MU-013382-01-0002) siRNAs using DharmaFECT (Dharmacon; Lafayette, CO) according to manufacturer's protocol. si*CONTROL* Non-targeting siRNA no. 2 was used as a control. Cells were collected 72 hrs after transduction for RNA or protein preparation.

Quantitative Real-time PCR

qRT-PCR was analyzed using the comparative ΔCt method (ABPrism software, Applied Biosystems; Foster City, CA) using *GAPDH* as an internal normalization control. Quantification is an average of at least 3 independent experiments and standard errors are indicated. Fold *Maspin* mRNA levels were expressed relative to control (cells transduced with an empty retroviral vector).

Western Blotting and Immunofluorescence

Transduced cells were lysed with RIPA Buffer (Sigma-Aldrich; St Louis, MO) for Maspin protein analysis and NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce Biotechnology; Rockford, IL) was used for SOX2 protein analysis. Western blotting was performed using standard methods. Antibodies included: anti-Maspin (BD Pharmingen; San Diego, CA) diluted 1:500; anti-Tubulin (Sigma-Aldrich; St Louis, MO) was diluted 1:5000; anti-UHRF1 (Abcam;

Cambridge, MA) diluted 1:1000; anti-SOX2 (Cell Signaling Technology; Danvers, MA) diluted 1:1000; and anti-H3 (Active Motif; Carlsbad, CA) diluted 1:10,000. For Immunofluorescence staining, transduced cells were grown on glass coverslips and stained according to standard methods. anti-HA (Covance; Princeton, NJ) antibody was diluted 1:000 and detected with Alexafluor-594 (Invitrogen; Carlsbad, CA) goat anti-mouse diluted 1:500.

Bisulfite Conversion, Cloning, and Sequencing

Bisulfite modification of genomic DNA was performed using a procedure adapted from Grunau et al [44], as described previously [45]. Cloning and sequencing of PCR products were described previously [46].

Anchorage-independent Growth and Cell Viability Assays

Anchorage-independent growth assays were performed using standard methods and evaluated visually for the presence of colonies that were greater than 2 mm in diameter. Cell viability was assessed by CellTiter Glo (Promega; Madison, WI) according to the manufacturer's protocol.

Demethylating Treatment with 5-aza-2'-deoxycytidine

5-aza-2'-deoxycytidine (5-aza) (Sigma-Aldrich; St Louis, MO) was employed as a demethylating agent. ZF-97 DNMT3a clones 10 and 12 were propagated in freshly made growth medium containing 1 μM or 5 μM 5-aza for 3 days, with re-feeding of fresh media containing the drug every day. Cells were collected after treatment for RNA preparation.

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Chapter 6

Stable inherited oncogenic silencing in vivo by programmable and targeted de novo DNA methylation in breast cancer

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Submitted to Cancer Research

ABSTRACT

The recent mapping of cancer epigenomes has unveiled large-scale hot spot perturbations in DNA methylation patterns relative to normal tissue. Armed with this knowledge, there is now a need for functional epigenomic approaches to edit the aberrant epigenetic state of key cancer drivers to precisely reprogram the epi-pathology of the disease. In this study we demonstrate that artificial, targeted incorporation of DNA methylation in the oncogene *SOX2* in breast cancer through a six-zinc finger protein linked to DNA methyltransferase 3A (ZF-DNMT3A) fusion leads to a permanent repression of oncogenic gene expression. The *de novo* DNA methylation was faithfully propagated and maintained through cell generations even after the suppression of the expression of the chimeric methyltransferase in the tumor cells. In xenograft studies in mice we demonstrate stable repression of *SOX2* expression and long-term tumor growth inhibition for more than 100 days post-implantation. In contrast, down-regulation of *SOX2* by ZF domains engineered with transcriptional repressor domains not possessing intrinsic enzymatic activity resulted in a transient and reversible suppression of oncogenic gene expression. Our results demonstrate that the targeted *de novo* DNA methylation is sufficient to induce stable oncogenic silencing and it is not only maintained during cell division but also significantly delays the tumorigenic phenotype of cancer cells *in vivo* even in absence of treatment. We outline a novel approach to permanently inhibit drivers overexpressed in cancer and other diseases.

SIGNIFICANCE

Artificial *de novo* methylation in specific onco-targets overexpressed in cancer cells resulted in stable oncogenic silencing, which was faithfully transmitted through cell generations and significantly inhibited tumor growth even in total absence of treatment. Our work suggests that the *de novo* DNA methylation *per se* acts as an "epigenetic trapping" mechanism sufficient to establish a program resulting in potent and stable heterochromatin formation and gene silencing. The discovery that *de novo* methylation is *per se* sufficient for the restoration of a quasi-perpetual, normal-like epigenetic state in oncogenic contexts opens the exploration of genome-based engineering approaches to target currently undruggable cancer drivers. Our approach can also be exploited for the locus-specific and dynamic control and functionalization of the epigenome in cancer and other model systems.

INTRODUCTION

Cytosine methylation in the mammalian DNA is a key epigenetic modification controlling essential processes, such as imprinting, X-chromosome inactivation, silencing of retrotransposons, cell fate and organism's development. In addition to DNA methylation, repressive histone post-transcriptional modifications reinforce gene inactivation resulting in the formation of mitotically inherited inactive chromatin, namely heterochromatin (1). Importantly, the epigenetic information must be stably inherited during mitosis for the proper maintenance of cellular identity, a process referred as epigenetic memory (2). In pathological states, such as cancer, the epigenetic landscape of normal cells becomes disrupted. Aberrant incorporation or removal of epigenetic modifications in cancer cells leads to genome-wide alteration of gene expression, including inactivation of tumor suppressor genes and reactivation of oncogenes (3).

Changes in DNA methylation patterns are characteristic hallmarks of the distinct intrinsic subtypes of breast cancer. Poorly differentiated, highly proliferative basal-like breast cancers associated with stem/progenitor cell-like features are significantly hypo-methylated relative to the other breast cancer subtypes. Importantly, these tumors are driven by aberrant activation of multiple developmental transcription factors (TFs), which fuel the tumor with sustained proliferation, drug resistance and metastatic capacity (4-6).

The High Mobility Group (HMG) oncogenic TF *SOX2* is normally expressed in embryonic stem cells (ESCs) and neural progenitor cells, where it maintains self-renewal. DNA methylation in the *SOX2* promoter and enhancer regions functions as an epigenetic switch which forces cells to undertake differentiation gene programs (7). *SOX2* is therefore not expressed in most normal adult tissues (7-9). However, aberrant reactivation of *SOX2* has been detected in ~43% of basal-like breast cancers and in several other malignancies, including glioblastomas lung, skin, prostate and ovarian carcinomas (10). *SOX2* overexpression in tumor specimens has been associated with promoter hypo-methylation relative to adjacent normal tissue and with copy number amplification (11, 12). The oncogenic role of *SOX2* in breast cancer has been associated to direct activation of *CYCLIN D1* resulting in increased mitotic index and proliferation (13, 14). Down-regulation of *SOX2* by RNAi decreased the tumorigenic phenotype in lung, breast and ovarian cancers (10, 13, 15). Moreover, a major obstacle of siRNA and shRNA approaches in long-term studies in animals has been the short half-life of the RNA or the methylation of the virally encoded shRNA promoter, which represent a very significant limitation for cancer therapy.

In light of the essential role of DNA methylation in the regulation of SOX2 we hypothesized that targeted *de novo* methylation in this oncogenic context would result in an epigenetic "off" switch, forcing cancer cells to undertake differentiation programs. Furthermore, because *de novo* methylation is read and written by endogenous proteins and faithfully transmitted during cell division, we reasoned that artificial incorporation of *de novo* methylation in the *SOX2* context would confer stable and inherited oncogenic silencing, resulting in a sustained blockade of cell growth, faithfully propagated during cell generations. The epigenetic reprogramming of an oncogene towards a "normal-like" state, which will be stably inherited through mitosis, represents a hitherto unexplored paradigm

in cancer research; the resulting therapeutic effect would provide durability and sustained anti-cancer response ("*memory*").

Herein we took advantage of an engineered TF targeting the *SOX2* promoter made of six zinc finger (6ZF) domains recognizing 18 base pairs (bp) sites in the core promoter. These 6ZFs were linked to the catalytic domain of DNA methyltransferase 3A (DNMT3A), an enzyme catalyzing *de novo* DNA methylation, to correct the aberrant methylation state of *SOX2* in cancer cells. We provide evidence that targeted *de novo* DNA methylation in an oncogenic locus is sufficient to initiate and even reinforce, potent and inherited gene silencing, resulting in epigenetic, transcriptional and phenotypic memory. Our approach could be utilized to promote customizable heterochromatization and perpetual silencing of elusive cancer drivers mapped by recent genomic projects for which no drug is currently available.

RESULTS

ZF598-DNMT3A initiates SOX2 down-regulation accompanied by stable inhibition of cancer cell growth.

To induce targeted DNA methylation in the *SOX2* locus we took advantage of our previously characterized 6ZF arrays designed to recognize 18 base pairs (bps) sequences in the *SOX2* proximal promoter (Figure 1A) (14). Subsequently, we engineered the catalytic domain of the human DNA methyltransferase 3A (DNMT3A) at the C-terminus of the 6ZF domains and in frame with a 13 amino acid flexible linker, the ZF598-DNMT3A fusion. The same 6ZF arrays linked to the repressor Krueppel-associated box (SKD, ZF598-SKD) was used as a control, since this domain promotes potent KAP1-dependent repression when linked at the N-terminus of the 6ZFs (14).

We chose the MCF7 breast cancer cells as a model cell line to study the temporal dynamics of incorporation DNA methylation hence these cells express high levels of *SOX2* and they harbour a hypo-methylated promoter relative to normal cells (Figure 2A). The 6ZF constructs were delivered into MCF7 cells using inducible retroviral vectors (14) and stable clones were isolated in which the temporal expression of the ZF598-DNMT3A and ZF598-SKD fusions was controlled by doxycycline (Dox) treatment.

Upon induction of the ZF598-DNMT3A and ZF598-SKD expression by Dox (+Dox) *SOX2* mRNA levels decreased by 90% and 73%, respectively, as compared with control (empty-vector) transduced cells (Figure 1B). When Dox was removed for up to eight days (~10 cell generations) from the culture media (R, Figure 1B-C) cells transduced with ZF598-SKD restored *SOX2* expression to levels that were similar to those of un-induced cells, while cells expressing the ZF598-DNMT3A showed a persistent and even higher decrease of *SOX2* expression (85% relative to un-induced cells) upon Dox-removal (Figure 1B). These findings demonstrate that the DNMT3A construct conferred transcriptional memory. The *SOX2* transcriptional silencing induced by ZF598-DNMT3A was accompanied by a significant down-regulation of SOX2 protein expression. This suppression was sustained in Dox-removal conditions in absence of expression of the ZF methyltransferase and even resulted in increased levels of SOX2 silencing relative to +Dox cells as demonstrated by Western blot (Figure 1C).

We next investigated if the ZF598-DNMT3A fusion was able to maintain a suppression of the tumor cell growth in Dox-removal conditions ("phenotypic memory"). We performed a time-course cell viability assay to monitor cell proliferation changes associated with induction and subsequent removal of the ZF protein expression (Figure 1D). MCF7 cells stably transduced with either empty vector control, ZF598-SKD or ZF598-DNMT3A were treated for 72 hours with Dox (+Dox) to induce the expression of the ZF proteins. Next, Dox was removed from the culture media (Figure 1D, red arrow) and cell proliferation was monitored for the next 144 hours. Cells expressing empty vector or ZF598-SKD restored their proliferation rates upon Dox-removal. In contrast, cells expressing ZF598-DNMT3A maintained a robust inhibition of cell proliferation after Dox-removal. These results demonstrate the unique capacity of the 6ZF-DNMT3A fusions to establish an oncogenic silencing state at both, mRNA and protein level, that was stably maintained and even reinforced through cell generations.

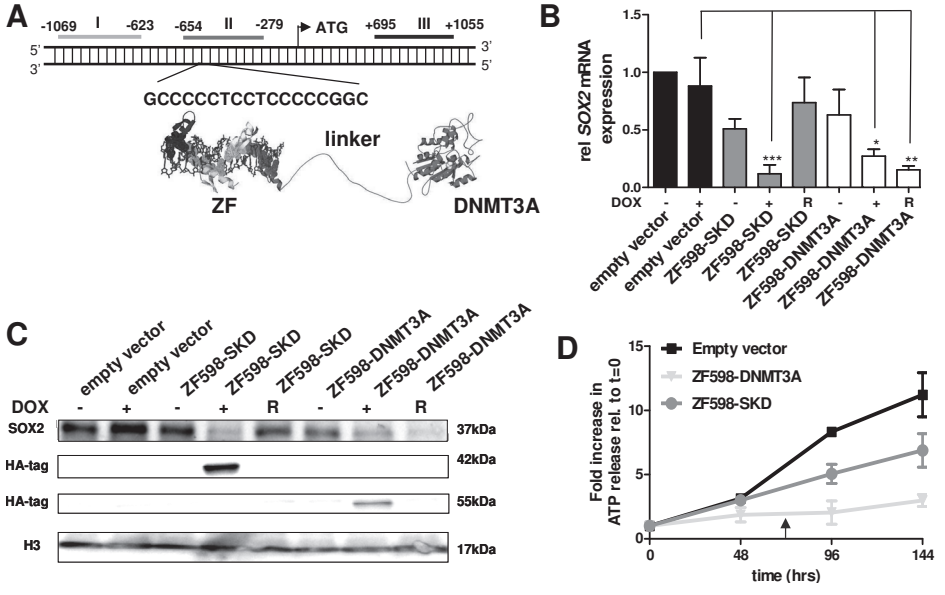

Figure 1. Stable down-regulation of the SOX2 expression by ZF598-DNMT3A. **A)** Schematic illustration of the *SOX2* promoter indicating the domain structure of the ZF598-DNMT3A construct, its binding site and the three amplicons analyzed by sodium bisulfite sequencing (amplicon I, light grey, II, dark grey and III, black). **B)** Quantification of *SOX2* mRNA expression by qRT-PCR in MCF7 cells. Cells were stably transducted with empty vector, ZF598-SKD and ZF598-DNMT3A. The expression of the ZF fusion was controlled by addition or removal of doxycycline (Dox); R = Dox-removal. Error bars represent standard deviation (SD) (***p < 0.001, **p-value < 0.01, *p-value < 0.05). **C)** Detection of SOX2 by Western blot. The C-terminal Hemagglutinin (HA) tag was used for immunodetection of the ZF proteins. An anti-histone H3 antibody was deployed as loading control. **D)** Cell viability of MCF7 cells assessed by Cell TiterGlo assays. Empty vector, ZF598-SKD and ZF598DNMT3A-transduced cells were induced with Dox for 48 hrs and removed from Dox after 72 hrs (arrow). *P* values between empty vector and ZF598DNMT3A and between ZF598SKD and ZF598-DNMT3A were $p<0.001$ and $p<0.05$ respectively, at 144 hours.

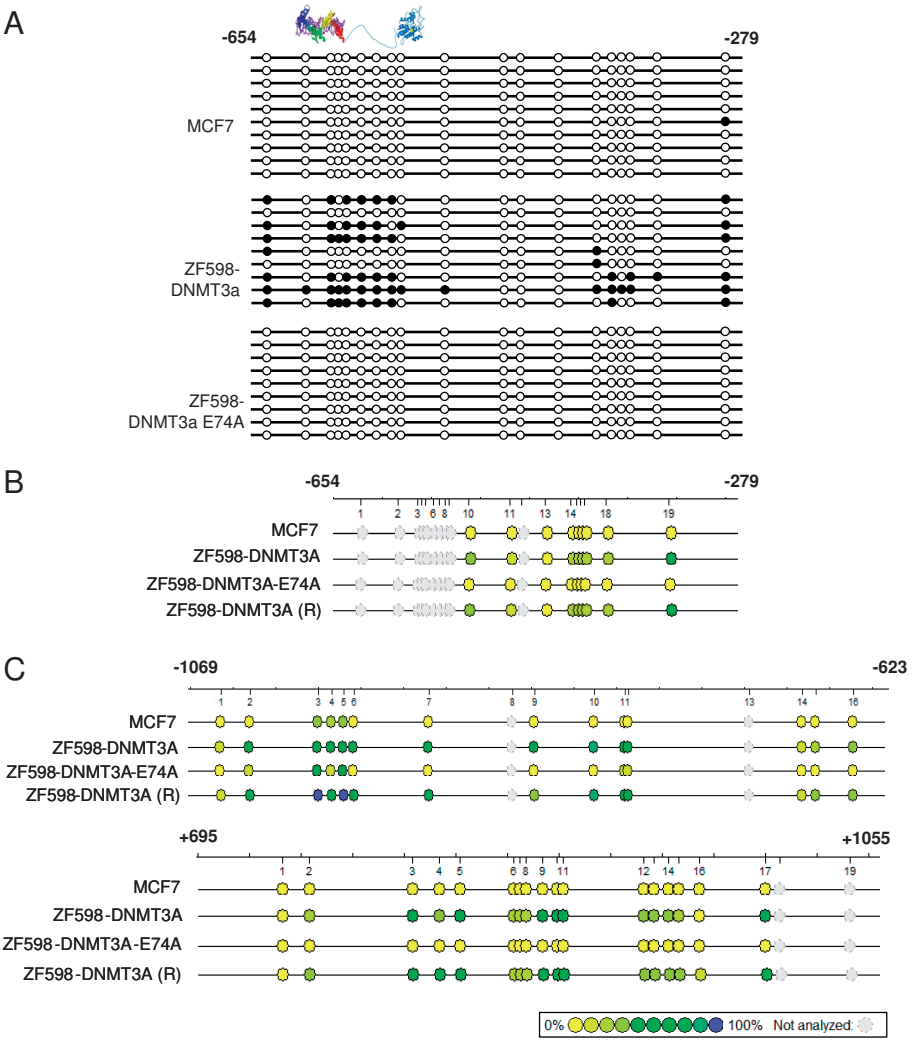

Figure 2. Expression of the ZF598-DNMT3A induces targeted DNA methylation in the *SOX2* promoter. **A)** Sodium bisulfite sequencing analysis of genomic DNA from MCF7 cells stably transduced with empty vector, ZF598-DNMT3A and ZF598-DNMT3A-E74A induced with Dox. Open circles refer to unmethylated CpGs and closed circles, methylated CpGs. **B)** MassARRAY analysis of amplicon II indicating average of DNA methylation frequencies for each CpG dinucleotide. Cells were first induced with Dox for 72 hrs and removed from Dox treatment (R). **C)** MassARRAY analysis of amplicons I (upper panel) and III (lower panel) after Dox-induction and Dox-removal (R).

ZF598-DNMT3A silences SOX2 by initiation and faithful propagation of DNA methylation

We next investigated whether ZF598-DNMT3A catalyzed targeted *de novo* DNA methylation within the *SOX2* promoter and flanking DNA regions. We harvested genomic DNA of MCF7 cells stably transduced with ZF598-DNMT3A and ZF598-DNMT3A-E74A (a mutant that abolishes catalytic activity (16)). Cells were treated with Dox to induce the expression of the ZF proteins. Next, cells were harvested at 72 hours and removed from Dox treatment (R) and passaged for additional eight days in Dox-free conditions to completely suppress the expression of the ZF protein. Genomic DNA was processed for sodium bisulfite conversion, followed by sequencing of several *SOX2* amplicons flanking the ZF-598 binding site. In addition, samples were subjected to MassARRAY to quantitate the incorporation of cytosine methylation along the promoter and flanking sequences. Three amplicons (from -1069 to +1055 bps relative to the translation start site; Figure 1A) were analyzed. Sodium bisulfite sequencing demonstrated that the amplicon containing the ZF598-DNMT3A binding site (-654 to -279 bps upstream of ATG) was free of CpG methylation in control and un-induced MCF7 cells (Figure 2A). This hypo-methylation of the *SOX2* promoter correlated with a high *SOX2* expression as assessed by qRT-PCR and Western blot (Figure 1B-C).

The induction of ZF598-DNMT3A expression resulted in an increase of DNA methylation of up to 90% at specific CpG dinucleotides (Figure 2A). Control cells or cells expressing the catalytic mutant ZF598-DNMT3A-E74A revealed no increase of CpG methylation, demonstrating that the induction of DNA methylation required the catalytic activity linked to the 6ZFs.

We next performed MassARRAY analysis to validate the *de novo* CpG methylation induced by ZF598-DNMT3A along the amplicon II before and after Dox-treatment and after Dox-removal (R) (Figure 2B). Consistent with the sodium bisulfite sequencing analysis, no methylation was detected prior to Dox treatment in both, ZF598-DNMT3A- and ZF-598DNMT3A-E74A transduced cells. Upon induction of ZF598-DNMT3A expression, DNA methylation levels dramatically increased 40-90% at specific CpGs flanking the ZF site, whilst no DNA methylation was detected in cells expressing ZF598-DNMT3A-E74A. Furthermore, both the pattern and intensity of the induced methylation were faithfully maintained over the time course of the experiment, even 8 days after Dox-removal (Figure 2B) when the expression of the targeted methyltransferase was not longer detectable by Western blot (Figure 1C).

To evaluate the spreading of DNA methylation in the *SOX2* context after the initial induction, we analyzed amplicons I and III encompassing 1 kb up- and downstream of the translational start site (Figure 1A and 2C). Sequencing analysis of amplicon I (-1069 to -623 bps upstream of the translation start site) demonstrated a dramatic increase of DNA methylation upon ZF598-DNMT3A expression relative to MCF7 or ZF598-DNMT3A-E74A cells (Figure 2C). The frequencies of DNA methylation in the amplicon I were significantly higher than those immediately adjacent to the ZF-binding site suggesting that the DNA methylation was quickly spread and reinforced in the regions flanking the 6ZF site. Most importantly, after removal of the ZF598-DNMT3A expression (R) the *de novo* methylation

increased additionally up to 100% at specific CpG sites. These results further support the enhanced down-regulation of *SOX2* expression after Dox-removal previously observed by qRT-PCR and Western blot analysis and outline the importance of CpG dinucleotides flanking the core promoter for the regulation of *SOX2* expression.

Lastly, the MassARRAY analysis in amplicon III (+695 to +1055 bps downstream of the translation start site) revealed no methylation in MCF7 cells. Upon ZF598-DNMT3A expression CpG methylation levels increased up to 90 % and as expected, no DNA methylation was induced in cells expressing the ZF598-DNMT3A-E74A mutant. Our time-course analysis indicate that the *de novo* DNA methylation induced by the artificial methyltransferase results in both, a phase of induction of gene silencing upon Dox-induction, and a phase of reinforcement of silencing (Dox-removal), which could be associated with propagation and spreading of DNA methylation from the 6ZF site during DNA replication. Furthermore, we demonstrate spreading of DNA methylation through at least 1 Kbp from the initiation site. Similar results were obtained deploying a second 6ZF protein (ZF552-DNMT3A) targeting an 18 bp site located at 552 bps upstream of the translation start site in the *SOX2* core promoter (Figure S1), which demonstrates that the approach is generalizable for other DNA-binding elements.

ZF598-DNMT3A expression confers anti-cancer memory and reduces tumor growth in a breast cancer xenograft in NUDE mice

To analyze whether the ZF598-DNMT3A construct induced phenotypic memory *in vivo* we took advantage of our inducible MCF7 cell lines stably transduced with either a catalytically active ZF598-DNMT3A or empty vector control. A total of $2x10^6$ MCF7 cells transduced with either ZF598-DNMT3A or control were implanted into the flank of nude mice and allowed to grow for 22 days before switching the animals to a Dox containing diet (Figure 3A). Within each group N=5 animals were maintained in a Dox-free diet. At day 19 post-induction, half of the ZF598-DNMT3A injected animals from the +Dox group were removed from the Dox-diet (N=10), to withdraw the expression of the ZF methyltransferase (R).

Over the period of 43 days, a significant inhibition (p= 0.0001) of tumor growth was detected in animals injected with ZF598-DNMT3A receiving a Dox containing diet (Figure 3B, right panel), which was superior to the inhibition we have reported for KRAB-containing repressors (14). Furthermore, this reduction of tumor growth was maintained up to 72 days after Dox-removal (p=0.001). Importantly, two animals induced with the ZF598-DNMT3A construct completely regressed tumor burden, not longer being detectable by caliper measurements. As expected, no significant change in tumor growth was detected in animals injected with empty vector (Figure 3B and 3C, left panel).

To study the stability of the therapeutic effect we removed the Dox from the diet of half (N=10) of the ZF598-DNMT3A animals (Dox-removal group), while maintaining the other half under +Dox conditions. These groups allowed us to investigate whether the tumor growth inhibition mediated by *SOX2* methylation was stably transmitted even after removal of ZF598-DNMT3A expression. Tumor volumes in the Dox-free, Dox-induced and Dox-removal groups were monitored for 43 days post-induction, which equals to 24 days post-removal of Dox for the Dox-removal group (Figure 3C, right panel). The tumor volumes

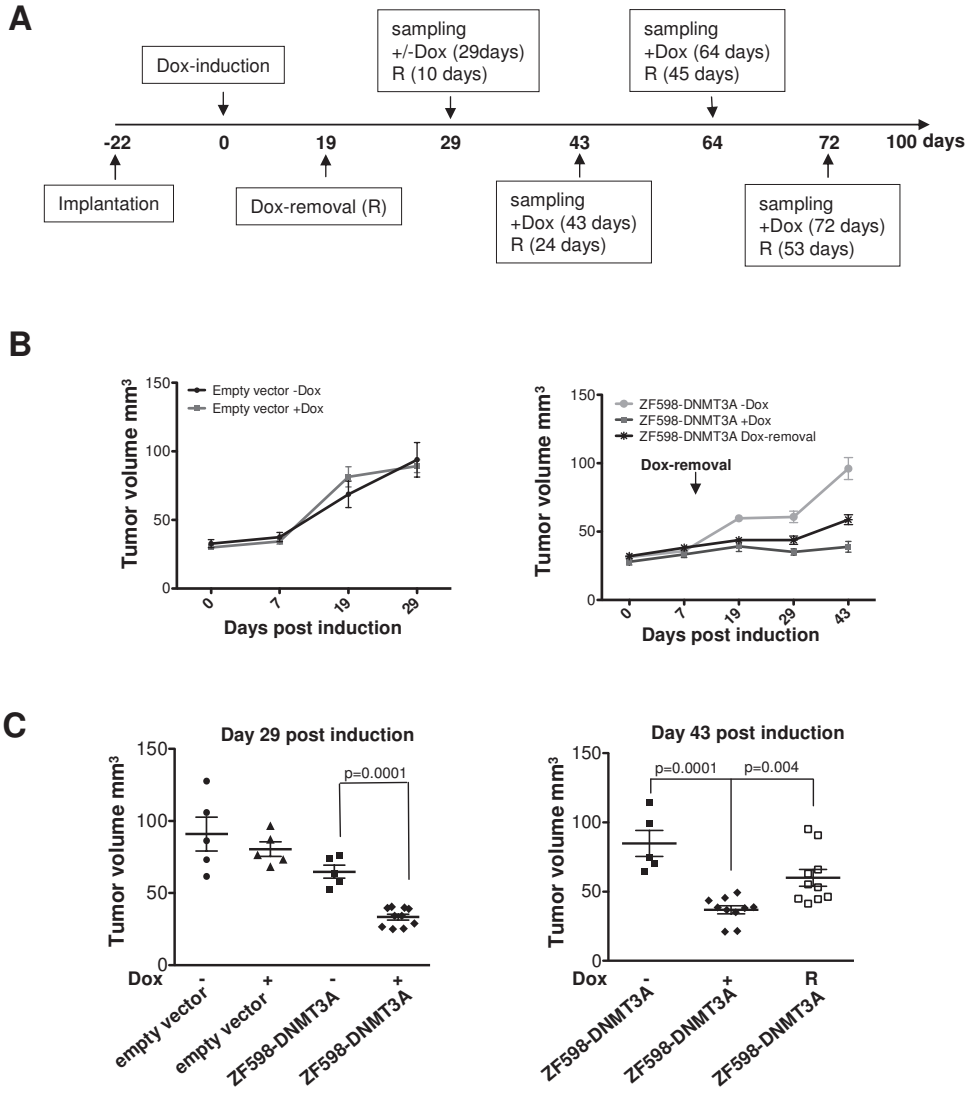

Figure 3. The *de novo* DNA methylation and oncogenic silencing induced by ZF598-DNMT3A are maintained long-term in a xenograft model of breast cancer. A) Time-line of the subcutaneous tumor injections of MCF7 cells in nude mice. B) Time course plot monitoring tumor volumes of empty vector control and ZF598-DNMT3A animals induced (+Dox) and un-induced (-Dox). Left panel: tumor growth of empty vector control animals. Right panel: tumor volumes of animals implanted with ZF598-DNMT3A. C) Left panel: tumor volumes of ZF-598DNMT3A and control animals at day 29 post-induction. Right panel: tumor volumes of ZF598-DNMT3A implanted animals at day 43, when the -Dox control tumors were collected ($p<0.05$).

of ZF598-DNMT3A +Dox animals demonstrated a significant inhibition relative to ZF598-DNMT3A -Dox animals (p=0.0001). Furthermore, mice removed from a Dox diet maintained a significant reduction of tumor volume (p=0.004) relative to un-induced animals (Figure 3C, right panel). However, the tumor sizes of mice removed from Dox slowly increased over time when compared with animals continuously placed under +Dox conditions. However, this effect could be due to the positive selection of cells carrying low levels of methylation in the xenografts or by functional compensation by another oncogenic driver.

To verify that the reduction of tumor growth in the ZF598-DNMT3A +Dox animals was due to the incorporation of DNA methylation, MassARRAY analysis of tumor DNA was performed at different time points post induction (Figure 4). DNAs extracted from empty vector +Dox tumors at 29 days post-induction were used as control. Upon induction of ZF598-DNMT3A expression an increase of DNA methylation was detected at all time points of sampling relative to control (Figure 3C). Importantly, this methylation was maintained *in vivo* for more than 50 days after Dox-removal in all samples analyzed (Figure 4). These data clearly demonstrated that the targeted DNA methylation was associated with tumor growth inhibition and with a significant decrease in the rate of tumor relapse when the treatment was discontinued.

Breast tumor growth inhibition was maintained upon clearance of ZF598-DNMT3A expression

To examine changes in the tumor morphology, hematoxylin-eosin stains were performed on sections of empty vector control and ZF598-DNMT3A tumors. The control tumors were collected 29 days post-induction, the ZF598-DNMT3A tumors were continuously induced with Dox for 72 days and for the ZF598-DNMT3A Dox-removal tumors, Dox was withdrawn for 53 days after an initial induction of 19 days. Histological analysis of empty vector +Dox

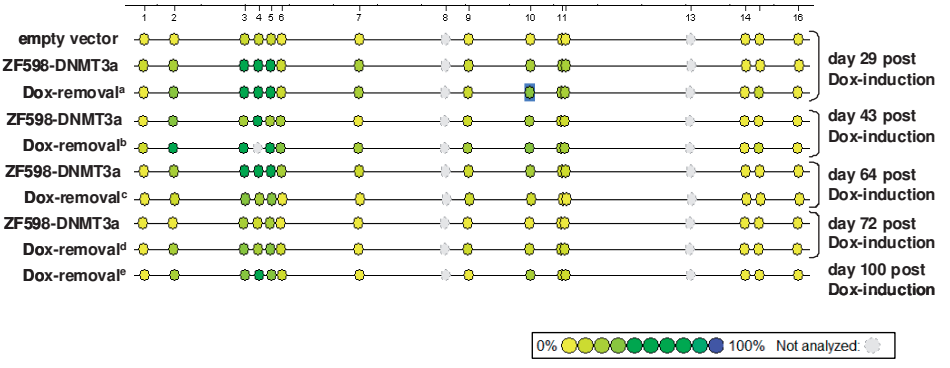

Figure 4. MassARRAY analysis of tumor DNA shows targeted induction and maintenance of DNA methylation. DNA from ZF598-DNMT3A +Dox and Dox-removal tumors was extracted at the indicated time points and subjected to sodium bisulfite conversion, followed by MassARRAY to detect DNA methylation frequencies. The amplicon I is located -1069 to -623 bps upstream of the translation start site. Each circle represents a CpG dinucleotide. Color code: yellow = un-methylated CpG, blue = 100% methylated CpG.

tumor sections revealed a high density of closely packed tumor cells at day 29 post induction (Figure 5A, left panel). In contrast, the ZF598-DNMT3A +Dox tumors exhibited a very striking change in the tumor architecture, with a more organized structure consisting of islands of tumor cells and an increase of intervening stroma, a phenotype that was maintained after removal of ZF598-DNMT3A expression (Figure 5A). This change in the tumor phenotype was reminiscent of a gain of normal-like epithelial features, with the formation of organized and more compact foci of tumor cells separated by stroma.

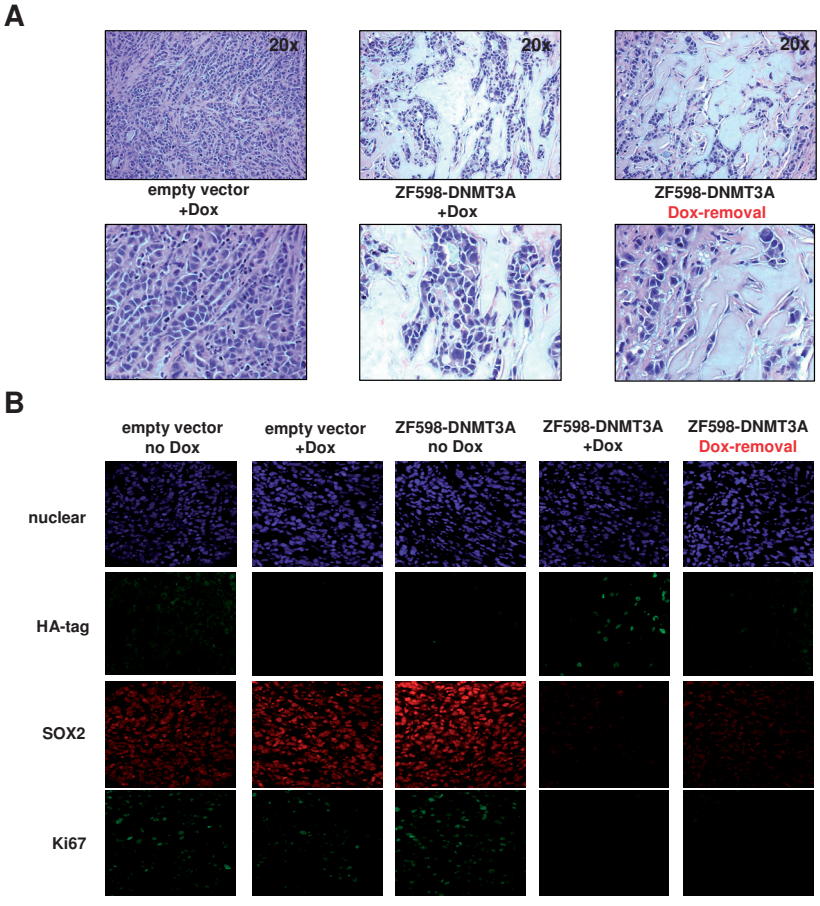

Figure 5. Histological and immunofluorescence analyses of tumor sections reveals phenotypic memory. A) Hematoxylin-Eosin stains of representative ZF598-DNMT3A -Dox, +Dox and Dox-removal tumor sections. Sections of empty vector tumors were extracted 29 days post induction, sections of ZF598-DNMT3A +Dox were sampled after 54 days of Dox induction and those of Dox-removal samples were harvested 45 days after Dox-removal. Pictures were taken at 20× and a detail of the image is shown. B) Immunofluorescence on sections of empty vector and ZF598-DNMT3A tumors collected 29 days post-induction. The expression of ZF598-DNMT3A (HA-tag, green), SOX2 (red) and the proliferation marker Ki-67 (green, bottom) in +Dox, -Dox and Dox-removal conditions are indicated. Images are taken at 40× magnification.

Immunofluorescence analysis of tumor sections revealed nuclear expression of the ZF598-DNMT3A protein in the +Dox group, but not in ZF598-DNMT3A -Dox animals (Figure 5B). This induction of methyltransferase expression correlated with a significant decrease in SOX2 expression in the tumors that received Dox, which was not observed in control (empty vector -/+Dox) and ZF598-DNMT3A -Dox tumors. After removal of Dox, the ZF598-DNMT3A expression was not longer detected in the tumor sections. Importantly, a decrease of SOX2 expression was stably maintained *in vivo* after Dox removal relative to control or -Dox tumors. In addition, the down-regulation of SOX2 expression correlated with a decrease in tumor cell proliferation, as indicated by the Ki-67 marker, which was maintained down-regulated relative to un-induced cells even after removal of ZF598-DNMT3A expression for 10 days (Figure 5B). These results suggest that the *de novo* methylation patterns were robustly maintained during somatic cell division *in vivo* resulting in the restoration of a more normal-like epithelial phenotype.

DISCUSSION

In cancer, aberrant DNA methylation is associated with initiation and progression of malignant disease. Like TFs, many oncogenic drivers in cancer are of undruggable nature, such as small GTPases (*KRAS*, *HRAS*). Therefore, to be able to program a hereditable targeted silencing state in these major oncogenic drivers would be of high impact in the cancer field.

Targeted DNA methylation by designer ZFs linked to the catalytic domain of DNA methyltransferases have been demonstrated by our group and others (16-18). Here, we show that DNA methylation targeted to the *SOX2* promoter significantly inhibits tumor growth in a xenograft mouse model of the breast. Furthermore, the *de novo* DNA methylation was stably maintained *in vivo* even 53 days after removal of the ZF598-DNMT3A and was accompanied with a sustained suppression of *SOX2* expression and tumor growth inhibition.

In this study we utilized a modular ZF protein engineered to bind an 18 bp sequence in the core promoter of *SOX2*. ZFs are well characterized DNA binding domains and have been used for almost two decades for DNA targeting with customizable sequence selectivity (19). However, novel approaches to target endogenous gene expression became recently available, such as transcription activator like effectors (TALEs) (20, 21) and the RNA-guided clustered regularly interspersed short palindromic repeats (CRISPR/Cas system) (22, 23). Both approaches could be used alternatively to ZFs to target the catalytic active domain of DNMT3A to specific chromosomal sites. Pioneering work has been recently published by Konermann *et al.*, who fused 32 different histone effector domains to a TALE DBD targeting the *Neurog2* locus and demonstrated transcriptional repression (24). However, the spatio-temporal dynamics associated with *de novo* DNA methylation and histone post-transcriptional modifications still remains elusive. Here we demonstrate that *de novo* DNA methylation is maintained during replication after removal of ZF-DNMT3A expression. We speculate that the artificial incorporation of DNA methylation provides an initial platform, which is read, written and propagated by endogenous epigenetic complexes during DNA replication.

The protein UHRF1 (ubiquitin-like PHD and RING finger domain containing 1) at the replication fork is absolutely required for the faithful transmission of methylated DNA (25,

26). UHRF1 binds to hemi-methylated DNA at the newly synthesized strand and directly interacts with DNMT1. In addition, UHRF1 also recruits the histone methyltransferase G9a and histone deacetylases (HDACs), which would then modify the newly resembled nucleosomes (27-29). Thus, the complex between DNMT1, together with UHRF1 and proliferating cell nuclear antigen (PCNA) at the replication fork would mediate the cross-talk between methylated DNA and repressive histone modifications (30, 31). In our experiments, it is conceivable that such cross-talk is responsible for the increased down-regulation of *SOX2*, hence histone modifications have been shown to reinforce the transcriptional memory of methylated genes in cancer (32). In addition to hereditable transmission during mitosis, we observed that the *de novo* methylation was rapidly spread at least 1 Kbps away from the ZF binding site. Potential mechanisms of genomic spreading involve chromatin looping and an iterative reading and writing of DNA and histone post-transcriptional modifications by the endogenous modifiers (33). The spatio-temporal propagation of DNA methylation and histone post-transcriptional modifications genome-wide in cancer cells is actively being investigated in our laboratory.

In contrast with DNMT3A, cells expressing the ZF-SKD fusion did not sustain down-regulation of SOX2, indicating mechanistically distinct epigenetic processes initiated by the ZF-DNMT3A and ZF-SKD constructs. First, recognition of methylated DNA by methyl-CpG binding proteins leads to recruitment of HDACs and chromatin remodelers (34). This could further reinforce the repressive state of the newly methylated *SOX2* oncogene. Second, SKD mediates silencing of target genes through recruitment of KAP1 (KRAB associated protein 1), which acts as a scaffold for heterochromatin-inducing modifiers such as HP1α and methyltransferase SETDB1 (35). However, SKD has not intrinsic enzymatic activity; acting mainly as recruiter, the suppression of SKD expression might entail a restoration of the euchromatic state and a re-priming of oncogenic transcription.

To date four epigenetic drugs have been approved by the U.S. Food and Drug Administration, including two DNMT inhibitors and two HDAC inhibitors (36). Both approaches aim to reactivate aberrantly silenced tumor suppressor genes. We report here an alternative strategy to current drugs enabling silencing of aberrantly expressed oncogenes. To deliver our ZF-DNMT3A fusions, nanoparticle technology is currently being developed. We have previously demonstrated systemic delivery of lipid-protamine-RNA (LPR) nanoparticles encapsulating mRNA encoding a ZF protein up-regulating the *MASPIN* promoter for the treatment of serous ovarian cancer (37). Such LPR technology could be similarly adapted to deliver the ZF-DNMT3A constructs for stable heterochromatization and epigenetic silencing of currently undruggable oncogenic drivers. The small, compact molecular architecture of ZF domains and their lack of immunogenicity make them very suitable molecular scaffolds for this type of delivery.

In summary, we demonstrate the applicability of ZF-DNMT3A fusions to induce targeted DNA methylation to stably repress target gene expression and tumor growth inhibition in a long-term xenograft mouse model of breast cancer. In addition to its important implications in cancer therapeutics, our approach provides a novel system to induce targeted DNA methylation and investigate the temporal and spatial propagation of this epigenetic state and its effects on gene expression on a genomic level *in vivo*.

EXPERIMENTAL PROCEDURE

Design of ZF-DNMT3A fusions and generation of stable cell lines

The construction of the SKD, DNMT3A, DNMT3A-E74A and the 6ZF domains has been described elsewhere (14, 17). The coding sequences of ZF598-DNMT3A and ZF598-DNMT3A-E74A were subsequently cloned in frame into pRetroX-Tight-Pur (CloneTech; Mountain View, CA). Generation of ZF598-DNMT3A and ZF598-DNMT3A-E74A stable expressing MCF7 cells was performed as described (14). Cells were induced with Doxycycline (Dox) every 48 hours and either harvested at 72 hours after first induction (+Dox) or removed from Dox (Dox-removal) and subcultured for additional 8 days under Dox-removal conditions. Real time PCR (qRT-PCR) and Western blot and immunofluorescence was performed as described (14).

Cell Proliferation Assay

For cell proliferation analysis 18 replicates of MCF7 cells stably expressing empty vector, ZF598-SKD and ZF598-DNMT3A were plated in 96-wells flat bottom plates with a density of 1000 cells per well. Twelve replicates were induced with Dox at time point 0 and after 48 hours. After 72 hours six replicates were removed from Dox, while six replicates were continuously induced. Cell proliferation was assessed by CellTiterGlo assay (Promega; Madison, WI) every 24hrs for a total period of 144hrs. Luminescence was detected in a PHERAstar plate reader and analysed using pherastar software. Results were normalized to readings obtain at day 0.

Bisulfite conversion and MassARRAYs

After genomic DNA extraction 2 µg of sample DNA (derived from either cell line or tumor), was treated with sodium bisulfite using the EZ DNA Methylation-Direct Kit (Zymo Research, Irvine, CA, USA). We custom designed primers for three amplicons spanning the core *SOX2* promoter, one specifically including the 6ZF binding site 5′-GCCCCCTCCTCCCCCGGC-3′ and two amplicons up- and down-stream of the 6ZF binding site. PCR was then carried out on 5-10 ng of sodium bisulfite (NaBi) converted sample DNA using conversion specific primers. For amplicon I: forward primer 5′-aggaagagagGGATAGAGGTTTGGGTTTTTTAATTT-3′ and reverse primer 5′-cagtaatacgactcactatagggagaaggctAAACCAACCTACCAACCACTAAAA-3′. For amplicon II forward primer 5′-aggaagagagAAAGGTTTTTTAGTGGTTGGTAGGT-3′ and 5′-agtaatacgactcactatagggagaaggctAAAACTCAAACTTCTCTCCCTTTCT-3′ reverse primer. For amplicon III: 5′-aggaagagagTTTTGGTATGGTTTTTGGTTTTATG-3′ forward primer and 5′-cagtaatacgactcactatagggagaaggctAATTTTCTCCATACTATTTCTTACTCTCC-3′ reverse primer with lower case letters representing the T7 tag sequences. Percent *SOX2* DNA methylation was quantified using mass spectrometry with the SEQUENOM EpiTYPER® T complete reagent kit (San Diego, CA). Conventional sodium bisulfite sequencing analysis was carried out as described before using 5′-AAAGGTTTTTTAGTGGTTGGTAGGT-3′ forward primer and 5′-AAAACTCAAACTTCTCTCCCTTTCT-3′ reverse primer for PCR amplification of bisulfite converted DNA (17)

Mouse experiments

Female NUDE mice (age 4 weeks) were purchased from Taconic Farms (Hudson, NY USA) and housed under pathogen-free conditions. The Institutional Animal Care and Use Committee (IACUC) at the University of North Carolina at Chapel Hill approved all experiments described herein. Estrogen pellets containing 2mg 17β-Estradiol (Sigma-Aldrich Corp. St. Louis, MO) and 8mg Cellulose (Sigma-Aldrich Corp. St. Louis, MO), were subcutaneously implanted in the animals 7 days prior of the injection of the cells. MCF7 cells (2×10^6) were collected and re-suspended with matrigel (BD Bioscience, San Diego, CA) 1:1 volume ratio in a total volume of 100 ml. The cell–matrigel mixture was injected into the mouse flank of N=11 mice for empty vector and N=22 for ZF598-DNMT3A. Tumor growth was monitored by caliper twice a week. When the tumor reached a size of approximately 25–30mm^3, Doxycycline (+Dox) was administered to the mice in the form of green food pellets (200 mg/kg of mice chow) for a period of 19 days. At day 19 post-induction half of the animals of each group were removed from Dox, while the other half was maintained under Dox diet. During the entire experiment the mice weight was monitored to ensure absence of toxicity. Animals were euthanized when tumor reached 100mm^3 (empty vector 29 days post-induction, ZF598-DNMT3A noDox 43 days post-induction and ZF598-DNMT3A Dox-removal at 72 days post-induction). Statistical differences between control and ATF animals were assessed by Wilcoxon Ranks Sum Test analysis.

ACKNOWLEDGEMENTS

This research was supported by NHI-NCI Grants 1R01CA125273, 3R01CA125273-03S1, 7R01CA170370 and DOD W81XWH-10-1-0265 to PB

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Chapter 7

Summary

Promoter DNA methylation plays an important role in controlling gene transcription and therefore contributes to the regulation of many biological processes. In cancer, genome-wide methylation patterns are highly disrupted and aberrant DNA methylation has been associated with initiation and progression of malignant disease.

AIM

The underlying aim of this thesis was the long-term silencing of an endogenous oncogenic promoter by targeted promoter DNA methylation using zinc finger proteins; a novel approach to silence endogenous gene expression.

CHAPTER 2

After a general introduction in Chapter 1, construction of ZF proteins by a method called modular design is described in Chapter 2. Precisely, step by step details are described, from cloning the DNA binding domain, to the *in vitro* validation of ZF protein binding to its target site, to the verification that the ZF protein fused to its effector modifies expression of the gene of interest in a reporter assay.

CHAPTER 3

Chapter 3 describes the process of screening a retroviral ZF library to identify ZF proteins able to regulate *EpCAM* expression from the endogenous locus. *EpCAM* expression is involved in both protection and progression of cancer development. To identify ZF proteins able to up-regulate *EpCAM* expression, the library expressing ZF proteins fused to the VP64 transactivator domain was screened, and in addition, to down-regulate *EpCAM* expression the library linked to the repressor SKD was screened. In total, three rounds of selection were performed: however, this did not lead to the identification of potent *EpCAM*-regulating ZF proteins. To optimise the functionality of the ZF proteins we previously designed and engineered to bind the EpCAM promoter, nucleotide sequences coding for the DBD were subcloned into retroviral expression vectors. After retroviral transduction of these rational designed ZF proteins an activation of *EpCAM* expression was detected in *EpCAM* negative cells. This was further validated by us and recently published.

CHAPTER 4

In order to achieve oncogene silencing, also the transcription factor *SOX2* was targeted using modular designed ZF proteins. Chapter 4 shows the strong efficacy of designer ZF proteins in the down-regulation of gene expression. As a proof of concept that *SOX2* can be targeted using ZF proteins, we first validated gene repression using the transient effector domain SKD. All expressed modular designed ZF proteins decreased *SOX2* expression significantly in two cell lines. We demonstrated that the ZF proteins indeed show strong binding to their target site in the chromatin context using ChIP analysis. Furthermore, repression of *SOX2* expression led to dramatic phenotypic changes in cancer cells, such as

a decrease in cell proliferation and the ability to form colonies in soft agar. We chose one of the ZF proteins to further validate the phenotypic changes seen in cell culture experiments in a xenograft mouse model of breast cancer. Therefore, the ZF protein was cloned into a Tet-ON inducible system, where the expression of ZF protein is controlled by Doxycyclin (Dox); removal of Dox will lead to discontinuation of ZF-DNMT3A and ZF-SKD expression, respectively. Expression of the ZF fusion protein upon Dox-induction revealed a strong inhibition of tumour growth in the mice. This was associated with *SOX2* silencing and a decrease in the proliferation marker *Ki67*.

CHAPTER 5

After validation that the designed ZF proteins bind to their target region, we set out to permanently silence gene expression by introducing DNA methylation using DNMT3A. The catalytic active domain of the human DNMT3A was cloned to the ZF proteins targeting *SOX2* and another cancer related gene MASPIN. In Chapter 5, for the first time the stable silencing of two endogenous targets -the tumour suppressor gene *MASPIN* and the oncogene *SOX2*- using ZF-DNMT3A fusions is demonstrated. As expected, silencing of *SOX2* led to inactivation of oncogene expression; while, silencing of the tumour suppressor *MASPIN* increased cell proliferation and led to a more aggressive phenotype. This exemplifies nicely the necessity to guide the DNMT3A to its specific target site using sequence specific DNA binding domains. Silencing of *SOX2* expression using the ZF-DNMT3A fusion was, as expected, more stable than repression using the SKD repressor. In the case of *MASPIN*, the silencing effect achieved with the ZF-DNMT3A fusion was stable for 50 cell generations. Importantly, this maintenance of DNA methylation was abolished by siRNA targeted knock down of UHRF1, a protein required for transmission of DNA methylation during replication. This demonstrates that the artificially introduced DNA methylation is maintained by the cellular machinery, just as it has been shown for natural occurring DNA methylation.

CHAPTER 6

The ability to silence gene expression by targeted DNA methylation in cell culture experiments led us to study the longevity of the introduced epigenetic mark in a xenograft mouse model. The ZF-DNMT3A fusion was cloned into the Tet-ON Dox-inducible system and its functionality was characterised in cell culture experiments. Again, the expression of ZF-DNMT3A had a more permanent effect on *SOX2* down-regulation, and decrease in cell proliferation, than ZF-SKD after the removal of Dox. Removal of ZF-SKD expression fully restored the expression of *SOX2* at the mRNA and protein levels; while, ZF-DNMT3A-mediated target gene methylation induced stable gene repression. This phenotype was further tested in a xenografts mouse model of the breast. Induction of ZF-DNMT3A expression led to an inhibition of tumour growth *in vivo*, which was maintained for several weeks even after removal of ZF-DNMT3A expression. Additionally, analysis of tumour sections by immunofluorescence staining demonstrated SOX2 protein down-regulation upon induction of ZF-DNMT3A expression, which was maintained 80 days after removal

of Dox. This down-regulation of SOX2 expression correlates with down-regulation of the proliferation marker Ki67, which was also maintained after removal of ZF-DNMT3A expression.

In conclusion, here we demonstrate for the first time that targeted incorporation of DNA methylation at a gene of interest leads to the silencing gene expression. Furthermore, we show that as an epigenetic mark, DNA methylation is stably transmitted during mitosis and maintains the repressive state of the target gene.

Chapter 8

Discussion and Future Perspectives

The ultimate goal of this thesis was to achieve the long-term silencing of an endogenous oncogenic promoter by directed introduction of DNA methylation at a predefined locus. To achieve this aim four major challenges had to be addressed: 1) identification of DNA binding proteins to effectively bind to endogenous promoters; 2) feasibility of modulating the expression of the endogenous target gene by ATFs; 3) silencing of the endogenous promoter can be achieved through targeted DNA methylation; and 4) the artificially incorporated DNA methylation mark is mitotically transmitted.

1) IDENTIFICATION OF ZF PROTEINS TO BIND EFFECTIVELY TO THE ENDOGENOUS PROMOTERS

There is a high abundance of ZF based transcription factors in eukaryotic genomes, and 3% of the human genes encode for ZF proteins of the subtype of Cys_2-His_2 (1). To date, the major focus of research on ZF proteins has been on their ability to recognise DNA; however, ZF proteins can also undergo protein-RNA and protein-protein interactions (2,3).

In this study, two methods were used to identify ZF proteins that are able to regulate endogenous promoters; the modular design and the screening of a ZF library (**Chapter 2** and **Chapter 3**). The modular design of a ZF protein is simple, but does not take the endogenous promoter accessibility into account (**Chapter 2**). Cell based selection from a ZF library (**Chapter 3**) results in ZF proteins that can access endogenous genomic sites in the investigated cell type(s) as described below.

Selection from a Zinc Finger Library

ZF libraries have been used in the past to identify ZF proteins able to modify expression of endogenous target genes (4). The *in vivo* library screening of ZF proteins takes advantage of the large number and variety of ZF proteins in the library. We aimed to identify ZF-based artificial transcription factors for both the transcriptional up- and down-regulation of EpCAM expression by screening a ZF library. Therefore, we took advantage of the cell surface expression of EpCAM, which makes it suitable for antibody sorting using fluorescence activated cell sorting (FACS). In our hands the *in vivo* selection of ZF proteins modulating the *EpCAM* promoter did not result in the isolation of functional DNA binding domains (**Chapter 3**).

A technical limitation of this study was the recovery of ZF proteins by PCR, using primers that recognise the flanking sequences of the ATF. Because of the homology of the linker sequences it is likely that recombination occurred, between the different fingers as well as between the linker-sequences of the ATFs, resulting in ZF proteins which lost their functionality. Additionally, it is important to keep in mind that the promoter accessibility of a gene is different in different cell lines: therefore, it is not guaranteed that one ZF protein isolated by the ZF library screening will have promoter accessibility to the same gene in another cell line. Indeed, there has been only limited success selecting ZF proteins from a ZF library for specific up-regulation of genes silenced in cancer (4). Only two out of ten intended target genes have been successfully modified using the ZF library (4,5). Overall, the

screening of an *in vivo* ZF library is a technically challenging process and is associated with high costs, which makes it unsuitable for most laboratories. This is in great contrast to other strategies developed to generate and identify potent ZF proteins, such as OPEN, CoDA, the so called "Sangamo method" (6-8) and the modular assembly of ZF proteins as further discussed in **Chapter 2** which are cheaper and experimentally less challenging to perform.

The modular assembly of Zinc Finger Proteins

An alternative method to the screening of a ZF library to obtain efficient ZF proteins is the "modular approach". The process of modular ZF assembly precedes an *in silico* selection, providing possible target sites available for ZF binding within the selected region (9). This is followed by a *NCBI BLAST* search to confirm the uniqueness of the selected target site within the genome. Sequences of ZF building blocks that will bind 5'-GNN-3', 5'-ANN'3 and 5'-CNN-3' nucleotide sequences are chosen from available lexicons and ZF fusion proteins are generated by modular design [(**Chapter 2** and (10-13)]. Overall the modular approach allows a relatively easy and cost efficient generation of several designer ZF proteins within one week.

Despite one study reporting an unexpectedly high failure rate of modular designed ZF proteins (14), successful gene targeting by modular designed ZF proteins has been shown on several exogenous and endogenous targets [**Chapter 3-6** and (15-18)]. The ZF proteins ZF-A and ZF-B in **Chapter 3** were generated by modular design and their activity after transient transfection on reporter constructs has been previously validated (19). It required the retroviral delivery of the ATFs into host cells to demonstrate their functionality at the endogenous promoter, as transient delivery did not regulate the endogenous gene expression [**Chapter 3** and (17)]. To target the endogenous *SOX2* promoter, four ZF proteins were designed and synthesized applying the modular approach. In our studies the modular design of ZF proteins showed a high success rate with three out of four ZF proteins being expressed and all three are highly functional in modulating the endogenous promoter *in vitro* and *in vivo*.

Binding specificity of designer ZF proteins

A major concern in the application of ATFs in gene therapy has been their sequence specificity. In the early 1990s, ZF DBDs were identified by phage display experiments in which proteins with high affinity to a target sequence were selected from phage display libraries (20). Using this technique ZF modules with the specificity to be able to discriminate even between closely related target sequences (GCG and GTG) were identified (21). Shortly after, the same group published the first study on repressing an endogenous target (the *bcr-abl* gene) using an engineered three finger ZF protein (22).

To increase genome-wide targeting specificity even further, ZF proteins with six finger modules were designed to target stretches of 18 nucleotides, which is mathematically unique in the human genome (23). A designed six finger ZF protein showed functional discrimination between the two endogenous binding sites which differ only in one base pair

(24). Importantly, increasing the size of the DNA recognition module does not result in an increased specificity of the DNA binding domain (DBD) (25), however increasing the length of the DBD does decrease the possible binding addresses in the human genome and this theoretically leads to a protein with higher specificity while providing higher binding affinity.

To predict the genome-wide binding specificity of engineered ZF proteins so far, mostly software-based tools have been employed (26,27). Experimentally, electro-mobility shift assays (EMSA), enzyme-linked immunosorbent assays (ELISA), reporter constructs and microarrays have been used to evaluate ZF protein-DNA interactions (19,28-31). EMSA analysis showed 60% to 100% sequence specificity of modular designed ZF proteins (28,29). Tan *et al*. reported in 2003 genome-wide single gene specificity by testing their modular designed ZF protein in an *Affymetrix U133 array*, which provides binding information on 22,225 probe sets which equals ~16,000 genes (32). To the best of our knowledge thus far no genome-wide binding study, such as ChIP-Seq analysis has been reported to map the binding sites of an engineered ZF protein (manuscript in preparation with Ryan Lister and Peggy Farnham). However, data obtained from a natural ZF-SKD protein analysis genome-wide by ChIP-Seq did show binding to more than 5000 genomic sites, as would be expected for a natural ZF protein (33). The majority (76%) of intragenic (40% of total peaks) binding sites lay within introns. Only 20% of the reported binding sites were located in close proximity to a transcriptional start site (TSS) (33).

Currently, the genome-wide consequences of intragenic ZF binding are unknown. For identified binding sites close to TSSs, it would be interesting to evaluate how many bound targets show differential target gene expression. Genome-wide ChIP-seq comparison of designer ZF proteins fused to SKD, DNMT3A and no effector domain, respectively, show multiple binding peaks (manuscript in preparation). All identified sites found to be bound by the ZF fusions show high sequence similarity to the intended target sequence; However, particularly ZF recruitment to CG-rich genomic regions was revealed, which mostly correlates with promoter sequences. A further significant finding was that the effector domain highly influences to which sites in the genome the ZF protein is recruited (manuscript in preparation). It is currently under investigation whether the genomic sites the ZFs are recruited to show epigenetic modifications and differential RNA expression.

Accessibility of the target gene promoter for ZF binding

The binding of designer ZF proteins to their recognition site is also dictated by the chromatin environment of the target gene. Crucial for successful modulation of endogenous gene expression is the promoter accessibility of the gene of interest for ZF binding. This is influenced by numerous factors at a given genomic site, including nucleosome positioning and occupancy by endogenous DNA binding proteins. Hypersensitivity of a genomic region to DNase I cleavage allows mapping of accessible sites within desired genomic target regions. Prior evaluation of DNAase I hypersensitivity sites is suggested to identify accessible regions for ZF binding. This may improve the success rate, although no clear rules have been distilled yet (23). Recently, the ENCODE consortium published data on genome-wide DNase I hypersensitivity sites of 125 human cell and tissue types (34). These openly

accessible data are valuable for researchers to identify possible target sites in their gene of interest. Furthermore, activating or repressive epigenetic marks at the target site might influence gene accessibility. For natural endogenous TFs, the chromatin formation at their binding site has a high impact on the ability of the TF to bind to its designated site. Similarly, these epigenetic marks could possibly interfere with the binding of designer ZF proteins: however, these epigenetic marks vary highly between cell/tissue types and sometimes even target sites that are close together can yield different activation/repression efficiencies, dependent on the (epi)genomic context [Chapter 5 and (16-18)]. To achieve successful gene expression modulation of the desired target gene it is advisable to generate several DBDs targeting different regions along the promoter site. In our studies two out of two 6ZF proteins engineered to target the *EpCAM* promoter and three out of four ZF proteins engineered to bind to the *SOX2* promoter showed successful modulation in gene expression; whereas, the "non-functional" *SOX2* ATF was not properly expressed [Chapter 3 and 4 and (17)].

As an alternative to ZF proteins, recently TALEs (transcription activator like effectors) and RNA-guided Cas9 (CRISPR-associated 9) DNA binding proteins have gained attention for gene targeting/editing (35,36). Dependent on their design, TALE DBDs can recognise 12 or more base pairs. Every targeting module of a TALE consists of 34 amino acid tandem repeats, where the 12th and 13th residue of each module makes contact with the DNA. However, because of the high sequence homology of the single repeats, the generation of TALEs appears to be more complex, as the single repeats tends to recombine during amplification and after delivery into target cells (37). Compared to ZF proteins, where each single module consists of 30 amino acids recognising three nucleotides, a single TALE repeat comprises 34 amino acids recognising only one nucleotide. This will result in an approximately three times larger DBD, which is a potential limitation for *in vivo* delivery and clinical applications (38). The Cas9 DNA binding proteins required for RNA guided gene targeting have a size of 150kDa and are therefore even larger than TALE proteins (~120kDa, depending on the number of repeats) for cellular and nuclear delivery. In this regard, the administration of the relatively small ZF proteins (~42kDa) *in vivo* has been successfully shown using nano-technology (39).

2) FEASIBILITY OF MODULATING THE EXPRESSION OF THE ENDOGENOUS TARGET GENE BY ATFS

To modulate gene expression of *EpCAM* and *SOX2*, and to determine effective binding of the ZF proteins, transient effector domains previously reported to affect gene expression were fused to *EpCAM* and *SOX2* targeting ZF proteins, respectively (Chapters 3 and Chapter 4). *EpCAM* expression has been shown by us and others to play a diverse role in cancers of different origin, and both specific up- and down-regulation, dependent on the tumor type, are desirable for therapeutic applications [reviewed in (40)]. Aberrant *SOX2* over-expression has been found in many malignancies and a close functional link to its role in stem cell maintenance has been reported (41,42). The expression of both genes is regulated by epigenetic mechanisms, involving DNA methylation (43). As epigenetic marks are mitotically stable, the overall aim in this study was the endogenous epigenetic modulation of *SOX2* and *EpCAM* gene expression. However, to prove that the intended

target genes indeed can be endogenously modulated, we directed the transient effector domains (SKD, krueppel associated box domain and VP64, a tetramer of the herpes simplex virus protein 16) to the *EpCAM* and *SOX2* promoters, respectively.

Targeted gene modulation using ZF proteins fused to transient effector domains

In the past, several effector domains have been described to modify gene expression when fused to ZF proteins (**Chapter 1, Table 1**). Amongst others, by far the most used, and therefore best described, transcriptional modifiers are the VP64 domain, used for targeted gene activation and the SKD traditionally used for transcriptional repression. Both the SKD and VP64 domain have no enzymatic activity by itself, resulting generally in a transient effect on gene regulation, by facilitating the recruitment of endogenous proteins to the target site. This results in transient changes of gene expression. It is noteworthy that the VP64 domain seems to be involved in the indirect induction of promoter demethylation, as shown by several independent research studies (16,44,45); however, this demethylation is not associated with permanent gene activation, possibly because inactivating histone modifications remain present at the target site. Experiments with doxycycline-controllable ZF-VP64 expression demonstrated that withdrawal of the ZF-VP64 expression (by removal of doxycycline) results in restoration of inactive gene expression (46). In that study, it was not assessed whether the re-silencing of gene expression was associated with a gain in DNA methylation of the target promoter. In similar experiments, we demonstrated that the ZF-SKD leads to a strong down-regulation; while, again, removal of ZF expression by depletion of doxycycline leads to a re-expression of *SOX2* (**Chapters 5** and **Chapter 6**).

One third of naturally occurring ZF proteins contain a SKD domain: therefore, SKD-containing ZF proteins constitute the largest group of transcriptional repressors in the genome of higher organisms (47). Historically, SKD has been identified as a transcriptional repressor of gene expression when fused to a GAL-4 DBD (48). Thereafter, SKD has been used as a repressor fused to designed ZF proteins in a multitude of targeting studies (**Chapter 1, Table 1**). The SKD-mediated repression is facilitated through recruitment of the co-repressors KAP1, which act as a scaffold for various histone remodelers such as HP1, NuRD, HDACs and SETDB1 (49-52). Within this complex HDAC1/2 functions as an eraser of histone acetylation and SETDB1 functions as a writer of histone methylation at lysine 9 (H3K9me). Both, deacetylated histones and H3K9me are correlated with repressive heterochromatin. Indeed, genes that are down-regulated through SKD exhibit an increased level of H3K9me3 and a decrease in H3-acetylation (53,54). However, irreversible SKD-mediated gene silencing (through DNA methylation) was only observed when SKD-targeting took place in very early mouse embryonic development (55). In differentiated cells SKD mediates strong gene repression but the effect is reversible upon SKD clearance [**Chapter 6** and (56,57)]. In mouse embryonic fibroblasts the established heterochromatin was shown to spread over the range of several kilobases (58). Indirectly, by the use of 5-azacytidine, we demonstrated in cancer cells that SKD-mediated silencing was not due to DNA methylation

as genome-wide inhibition of DNA methyltransferases had no effect on the silenced *SOX2* expression (**Chapter 4**). Instead of DNA methylation, ChIP analysis on cells with ZF SKD-induced down-regulation of *SOX2* expression showed loss of histone acetylation (manuscript in preparation with Peggy Farnham).

3) SILENCING OF ENDOGENOUS GENE EXPRESSION THROUGH TARGETED DNA METHYLATION

Previous studies gave great evidence that targeting methylation to DNA sites using bacterial and human DNA methyltransferases will result in gene repression [reviewed in (59)]. Most of these studies focused on the induced methylation of exogenous targets, including reporter plasmids and viral DNA, or mitochondrial DNA and sequences artificially integrated at chromosomal sites (60-65). In this thesis, the directed DNA methylation to a predefined endogenous target is shown to result in a down-regulation of gene expression (**Chapters 5** and **Chapter 6**). Comparison of ZF-DNMT3A and ZF-SKD mediated gene repression revealed that both fusion proteins induce strong transcriptional repression (**Chapter 6**). Although the ZF-SKD fusion shows a stronger immediate repression than the ZF-DNMT3A fusion, SKD repression is transient, and previous expression levels of *SOX2* are restored after removal of ZF-SKD expression (**Chapter 6**).

It is well documented that DNA methylation plays an important role in permanent gene silencing and that established DNA methylation is maintained during cell divisions to achieve stable gene repression (66-70). We demonstrated that targeting DNA methylation to an oncogenic promoter by ZF-DNMT3A did result in transcriptional silencing (**Chapter 5** and **Chapter 6**). The induction of DNA methylation accompanied by a reduction of *SOX2* and *MASPIN* expression indicates that targeted DNA methylation at both loci inhibits gene expression initiation. Importantly, in the SOX2 context, DNA methylation did not rapidly shut down transcription compared to SKD but the silencing effect of DNA methylation on gene repression increased over time (**Chapter 6**). This not only suggests that DNA methylation is maintained over cell generations, but also that cell divisions are required after *de novo* methylation to increase methylation associated gene silencing. The increased DNA methylation is most likely mediated through the accumulation of repressive histone marks established after replication. Therefore, formation of transcriptional silent heterochromatin upon targeted DNA methylation seems to require replication. Subsequently, DNA regions marked by ZF-DNMT3A mediated DNA methylation undergo heterochromatinization by removal of acetyl groups and H3K9me, which reinforces transcriptional silencing. As also described for the inactive X-chromosome and regions of genomic imprinting, DNA methylation is associated with stable gene silencing. In the context of the *MASPIN* promoter, DNA methylation was detectable for more than 50 generations in cell culture experiments (**Chapter 5**), and for *SOX2* for more than 50 days *in vivo* (**Chapter 6**). This further supports the notion that DNA methylation is associated with permanent gene silencing (71).

During preparation of this thesis Siddique *et al.* published a study using a ZF protein linked to a DNMT3A-DNMT3-like (DNMT3L) fusion targeting the VEGF-A promoter, which showed down-regulation of VEGF-A through targeted DNA methylation (72). This

furthermore confirmed the feasibility of artificially induced DNA methylation to mediate endogenous gene silencing. Interestingly, the ZF-DNMT3A -DNMT3L fusion had higher catalytic activity than the ZF-DNMT3A, even though DNMT3L itself has no methylation activity and merely guides DNMT3A to DNA by binding H3 only when H3K4 is unmethylated (72-74).

As mentioned above, repressive marks on histone tails are at least initially involved in the transcriptional silencing of gene expression, as demonstrated by the effects of SKD. The importance of histone modifications in targeted gene repression was further nicely exemplified by targeting studies of G9a linked to a ZF protein, directed against VEGF-A and Her2/neu respectively (75,76). Both studies showed targeted H3K9 methylation results in transcriptional silencing of target gene expression. It would have been interesting to see whether the direct methylation of H3K9 results in DNA methylation. In addition, very recently, the Church lab published a study showing down-regulation of gene expression after targeting 32 epigenetic modifiers fused to TALE DBD recognizing the *Grm2* and the *Neurog2* locus (77). Despite the affect on transcription, the study of Church *et al*. did not address if the down-regulation was due to epigenetic modifications being anticipated. Studies like this, however, do help to further broaden our understanding of epigenetic mechanisms in gene regulation.

4) THE ARTIFICIALLY INCORPORATED DNA METHYLATION MARK IS MITOTICALLY TRANSMITTED

DNA methylation is believed to play a critical role in permanent inactivation of gene expression (71,78). Furthermore, DNA methylation also occurs as a secondary event on genes which are already transcriptionally silent (79,80). The stable propagation of artificially induced DNA methylation through cell cycle has been shown previously at integrated chromosomal loci (65,81). One key finding of this thesis is that targeted DNA methylation at an endogenous target in an oncogenic context is sufficient to induce gene silencing and that the induced DNA methylation mark is also transmitted in *in vivo* situations (**Chapter 6**). Importantly, silencing of the target gene expression was stably propagated even after clearance of the ZF-DNMT3A fusion protein, indicating that DNA methylation in cancer cells critically contributes to long term gene silencing (**Chapter 6**).

The maintenance of DNA methylation over time suggests the involvement of DNA methyltransferase-1 (DNMT1) as well as DNMT3A and DNMT3B (82-85). For the reliable DNMT1-mediated methylation maintenance through replication, the association of DNMT1 with UHRF1 (ubiquitin-like PHD and RING finger domain-containing protein 1) is required (86). UHRF1 is a multi-protein complex consisting of RING (really interesting new gene), SRA (SET and RING associated) domain, UBL (ubiquitin-like) and PHD (plant homeo domain), which is able to bind to chromatin. The chronological order of events is not understood: however, a model suggests that the SRA domain of UHRF1 recognises and binds to hemi-methylated DNA, followed by recruitment of DNMT1, histone deacetylase-1 (HDAC1) and the histone methyltransferase (HMTase) G9a (87-91). Indeed, knock-down of UHRF1 in ZF-DNMT3A transduced cells abolished *de novo* methylation completely at the *MASPIN* promoter (**Chapter 5**). In the *SOX2* context, the additional down-regulation of

SOX2 expression after replication (**Chapter 6**) could also be due to the presence of factors involved in the formation of heterochrimatin at the replication fork. A higher abundance of UHRF1 after Dox-removal, however, was not validated by ChIP experiments (unpublished data). Further studies are required to identify the protein(s) involved in the transmission of artificially induced DNA methylation.

Previously, it has been shown that upon directing DNA methylation mediated by the bacterial DNA methyltransferase M.HpaII to a reporter sequence, which was integrated into mammalian DNA, the target site showed increasing levels of H3K9 methylation and a decrease in the activation mark H3K4 methylation (65). Therefore, it is likely that following *de novo* DNA methylation (induced by the ZF-DNMT3A fusion), H3K9 methylation takes place forming compact heterochromatin and subsequent transcriptional repression (92).

Tumour growth inhibition by targeted epigenetic oncogene silencing in vivo

Tumour cells show a significant distortion in their epigenetic landscape compared to that of normal cells (93-95). Hallmarks of the cancer epigenome are global changes in DNA methylation and histone modification patterns, as well as aberrant expression of histone modifying enzymes (96-99). The genome-wide alteration of the epigenetic state leads to the inactivation of tumour suppressor genes and also promotes tumourigenesis through the activation of oncogenes and genes involved in pluripotency (100-103).

SOX2 is a major pluripotency marker, highly expressed in embryonic stem cells, but undergoes epigenetic silencing upon differentiation (43). Therefore, *SOX2* is not expressed in most normal adult tissues. In contrast, over-expression of *SOX2* has been reported to promote tumour growth in many malignancies, including lung (104-108), breast (42,109-111), skin (112,113), prostate (114,115), ovarian (116-118) and sinonasal carcinomas (119). By directing DNA methylation through DNMT3A fused to a ZF protein targeting *SOX2,* silent *SOX2* expression was established in breast cancer cells and in a xenograft mouse model of breast cancer (**Chapter 6**). The Tet-ON inducible system allowed the controlled expression of ZF-DNMT3A fusion by administration of Doxycycline (Dox). Removal of Dox results in a discontinuation of ZF-DNMT3A expression. In our xenograft experiments, induced DNA methylation of the *SOX2* promoter resulted in down-regulation of *SOX2* and an inhibition of tumour growth. Furthermore, the induced DNA methylation and down-regulation of *SOX2* was propagated *in vivo* over cell generations and remained detectable even more than 50 days after removal of ZF-DNMT3A expression (**Chapter 6**). Overall, however, there was a decrease in the methylation level over time in both the Dox-removal group and the group continuously expressing the ZF-DNMT3A fusion. One possible reason for the reduction in the detectable methylation levels is that down-regulation of *SOX2* expression results in cancer cell death. Therefore, cells which were not sensitive to *SOX2* silencing retained the tumourigenic phenotype and possibly overgrew the population of cells with methylation-mediated *SOX2* silencing. This loss of DNA methylation is associated with a slight increase in tumour growth over time, in both the Dox-removal group and the group kept under Dox conditions. As active DNA demethylation enzymes have been described, another possible

reason for the decrease of DNA methylation is the active demethylation of the *SOX2* promoter (120,121); however, reactivation of SOX2 expression was not detected in this study.

In order to improve the epigenetic oncogene silencing, by inducing a faster and even more stable down-regulation of target gene expression, it would be conceivable to design ZF fusion containing a DNA-methyltransferase and a second epigenetic modifier. Indeed a multitude of epigenetic enzymes have been explored in targeting studies fused to different DBD [reviewed in (59)]. In particular, the HMTase G9a has been shown in two independent ZF targeting studies to induce H3K9 methylation and subsequent down-regulate target gene expression (75,76). Another powerful fusion partner than DNMT3s might be UHRF1, as it has the ability to recognise and function on hemimethylated DNA, thereby recruiting a multitude of epigenetic modifiers, possibly inducing an immediate formation of transcriptionally silent heterochromatin.

ZFP delivery

To deliver ZF proteins into their target cells different strategies have been employed, including viral transduction and plasmid transfection (**Chapter 3** to **Chapter 6**). For fast dividing (cancer) cell experiments in tissue culture, the most suitable delivery method has been, in our experience, retroviral transduction. In contrast to plasmid DNA, retroviral delivered DNA is integrated into the genome and therefore is maintained after cell divisions. Plasmid DNA is diluted out through cell division as it is not replicated along with the host cell DNA. For slow- or non-dividing cells lentiviral vectors have been used as a delivery method, because they have high infection capabilities in slow/non-dividing cells and the ability to integrate and their cargo is passed on during cell division. In clinical applications, however, the integration of DNA in the host genome is highly undesirable, as the integration site is mostly unpredictable and insertional mutagenesis due to integration can cause the development of cancer (122). Although integration-deficient adeno-associated viruses have been used in a wide range of clinical trials, in our hands delivery of ZF-SKD using AAV subtype 2 into MCF7 breast cancer cells did not result in suitable transduction efficiency [(123) and unpublished data]. Viral vectors have been mostly designed to infect a large variety of cell and tissue types with reduced specificity. To be clinically applicable, however, it is desirable to have delivery systems that do not result in integration into the host genome and that have a high specificity for the tissue or cell type of interest. Untargeted nanoparticles take advantage of the enhanced permeability and retention effect due to leaky vasculature of the tumour (124). Recently, we reported sigma receptor 1 targeted nanoparticle delivery of ZF protein-coding mRNA (39). In addition, protein delivery of ZF-nucleases has been shown to be successful (125).

FUTURE WORK

The successful silencing of oncogene expression by targeted incorporation of DNA methylation using ZF-DNMT3A fusion proteins opens the way for a multitude of new applications and directions for further basic research. Of most interest would be to investigate genome-wide DNA binding specificity of the artificial ZF proteins using ChIP-Seq analysis

(manuscript in preparation with Peggy Farnham) and their effects on DNA methylation patterns using whole genome bisulfite sequencing analysis (manuscript in preparation with Ryan Lister). Preliminary data of ChIP-Seq analysis show that the ZF proteins designed to target *SOX2* bind predominantly to the *SOX2* promoter however, additional regions with high sequence similarity also show binding peaks. This could be due to the relatively high expression level of ZF-DNMT3A after Dox-induction. Normally transcription factors are expressed at relatively low levels. The over-expression of ZF-DNMT3A in our Dox-inducible system has possibly led to a high abundance of ZF-DNMT3A, which could be the reason for the additional identified target sites: therefore, expressing the ZF-DNMT3A fusions at lower levels could help to prevent off-target sites occurring. Furthermore, our ChIP-Seq data indicate a distinct binding pattern of the ZF proteins to target regions dependent on the fused effector domain: the SKD fusions showed different preferred target sites than the ZF-DNMT3A fusions or ZF DBD alone. Comparison of ChIP-Seq binding sites to preliminary analysis of differentially methylated regions after ZF-DNMT3A expression shows *de novo* DNA methylation occurs at sites bound by the ZF protein. Complete data analysis will be necessary, however, to confirm these observations.

Another approach to increase specificity of targeted DNA methylation is the application of split enzymes (126,127): The DNMT3A enzyme is split into two fragments and each fragment is fused to a ZF protein, both of which are designed to bind in close proximity within the target gene. Binding of the two ZF proteins to their target region leads to assembly of the DNMT enzyme, restoring its catalytic activity and enabling site specific DNA methylation.

It would be very interesting to evaluate how targeted DNA methylation through the expression of the ZF-DNMT3A constructs modulates histone modifications within the *SOX2* locus. Furthermore it would be important to evaluate if the above mentioned off-target sites shown by ChIP-Seq and whole-genome bisulfite sequencing analysis appear to be permanently silenced through reinforced histone marks. The genome-wide analysis of ZF-DNMT3A induced *de novo* DNA methylation will give a deeper understanding of how epigenetic events spread upon incorporation. As DNA methylation is most likely reinforced by biochemical changes on histone tails, it would be interesting to evaluate the time and order in which these modifications occur.

As many other diseases, such as Multiple Sclerosis and Systemic lupus erythematosus, are caused by the loss of DNA methylation, ZF proteins, TALEs and gRNA Cas9 DNA binding proteins possibly could each be used to incorporate DNA methylation at specific sites for maintained silencing of their expression. Furthermore, this technique could be used in reprogramming studies, where targeted DNA methylation is of high significance in controlling differentiation.

In order to move targeted DNA methylation using designer DBD towards more clinically relevant applications, their specific delivery into the tissue of interest has to be improved. Previously promising results were obtained upon delivery of mRNA coding for ZF-fusions using nanoparticle delivery. This could be further optimised by coating nanoparticles with antibodies that specifically recognize the target tissue.

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Appendices

Nederlandse Samenvatting

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Acknowledgements

NEDERLANDSE SAMENVATTING

DNA methylatie speelt een belangrijke rol in het reguleren van genexpressie en is essentieel in de regulatie van veel biologische processen. In kankermateriaal worden over het gehele genoom grote afwijkingen in DNA methylatiepatronen gevonden. Deze verstoorde DNA methylatie wordt in verband gebracht met initiatie en progressie van maligniteiten.

Doelstelling van dit proefschrift

In dit onderzoek is getracht om DNA methylatie te gebruiken om de expressie van oncogenen langdurend stil te leggen. Voor dit doel is een innovatieve benadering gebruikt waarbij een DNA methylerend eiwit aan een DNA bindend eiwit (een zogenaamde zink vinger eiwit) wordt gekoppeld. Zink vinger eiwitten zijn zo gemaakt dat ze een sequentie in de promoter van het gen van interesse herkennen en daar aan binden, waarna het DNA methylerend eiwit deze promotersequentie zal methyleren.

Na een algemene introductie in **hoofdstuk 1**, wordt een zogenaamde "modulaire constructie" van zink vinger eiwitten beschreven in **hoofdstuk 2**. Stap voor stap worden alle details van klonering tot *in vitro* validatie van zink vinger eiwitten beschreven, inclusief het uitlezen van het effect van de zink vinger-effector constructen op de expressie van reporters. **Hoofdstuk 3** beschrijft een alternatief proces om zink vinger eiwitten specifiek voor het gen van interesse in handen te krijgen, namelijk het screenen van een retrovirale zink vinger constructen bibliotheek. In dit hoofdstuk is het Epithelial Cell Adhesion Molecule (*EpCAM*) het gen van interesse. Afhankelijk van het tumor type is *EpCAM* betrokken bij bescherming of juist bij progressie van kanker. Om dit beter te kunnen onderzoeken hebben we middels het retrovirale screeningsprotocol getracht zink vingers die efficiënt aan *EpCAM* sequenties binden te identificeren. Helaas heeft dit geen zink vingers opgeleverd en dus geen DNA bindende domeinen waar vervolgens transcriptiemodulatie domeinen aan gefuseerd kunnen worden om de expressie van EpCAM mee te moduleren. Om toch dergelijke "Artificiële Transcriptiefactoren" (ATFs) te verkrijgen, zijn eerder in ons lab ontwikkelde *EpCAM* bindende zink vinger eiwitten gekoppeld aan transcriptiemodulatoren en in een retrovirus gekloneerd om efficiëntie van ATF-behandeling te verhogen. Na infectie van *EpCAM* negatieve cellen met een retrovirus om *EpCAM*-bindende zink vinger eiwitten gekoppeld aan een transcriptie-activator tot expressie te brengen, werden deze negatieve cellen daadwerkelijk *EpCAM* positief. Deze bevinding met rationeel modulair geconstrueerde zink vingers is verder gevalideerd en recentelijk door ons gepubliceerd.

Om te onderzoeken of we de expressie van een oncogen stil kunnen leggen, zijn in **hoofdstuk 4** zink vinger eiwitten gemaakt, middels de modulaire design methode, om aan sequenties van het oncogen *SOX2* te binden. De zink vingers eiwitten zijn aan een transcriptierepressie domain gekoppeld (SKD) om te kunnen controleren of dit *SOX2* expressie verlaagt. Alle zink vinger constructen die goed tot expressie werden gebracht na retrovirale transductie verlaagden de *SOX2* expressie significant in twee cellijnen. Met behulp van ChIP analyse konden we inderdaad aantonen dat de zink vingers in de chromatine context aan de sequenties van *SOX2* binden. De afname in *SOX2* expressie resulteerde

in een dramatisch fenotypisch effect in kankercellen. Deze fenotypische veranderingen hebben we gevalideerd in een muizen model voor borstkanker. Hiertoe hebben we een zink vinger construct gekloneerd in een zogenaamd induceerbaar Tet-ON systeem, waarin de expressie van het zink vinger construct gereguleerd wordt door Doxycycline (Dox); stopzetting van de behandeling met Dox zal de expressie van het zink vinger construct doen stoppen. Expressie van een van beide constructen (zink vingers gekoppeld aan een DNA methyltransferase, ZF-DNMT3A of aan een transcriptie repressor, ZF-SKD) ten gevolge van Dox behandeling liet een sterke daling van kankercelgroei zien in de muizen. De groeiremming was geassocieerd met vermindering van *SOX2* expressie en een afname in de proliferatiemarker *Ki67*.

Na de validatie dat de modulair ontwikkelde zink vingers inderdaad aan hun target sequentie binden, hebben we getest of we de genexpressie blijvend kunnen beïnvloeden door DNA methylatie te introduceren met behulp van DNMT3A. Het katalytisch domein van het menselijke DNMT3A werd gekloneerd als fusie eiwit aan de *SOX2*-bindende zink vinger eiwitten en aan zink vinger eiwitten die het tumorsuppressor gen, *MASPIN*, binden In **hoofdstuk 5** hebben we als eersten kunnen laten zien dat remming van expressie van twee endogene genen - *MASPIN en SOX2*- inderdaad mogelijk is door middel van gen-gerichte DNA methylatie. Zoals verwacht resulteerde deze gerichte DNA methylatie in remming van expressie van *SOX2* en *MASPIN*. De remming van *MASPIN* expressie resulteerde in toegenomen celdeling en in een agressief tumor phenotype. Deze bevinding illustreert de noodzaak om het DNMT3A domein specifiek te laten aanhechten op zijn specifieke doel gen.

Het remmen van *SOX2* expressie met de ZF- DNMT3A fusie was, zoals verwacht, stabieler dan remming met de SKD repressor (**hoofdstuk 5**). Voor *MASPIN* was de remming van expressie ten gevolge van expressie van de DNMT3A-fusie stabiel gedurende 50 cel generaties. Na siRNA-knock down van UHRF1, een eiwit dat nodig is voor de kopiering van DNAmethylatie tijdens DNA replicatie, is het stabiele effect van DNA methylatie afwezig. Dit toont aan dat de kunstmatig geïntroduceerde DNA methylatie wordt onderhouden door de cellulaire machinerie, zoals ook gebeurt bij natuurlijk voorkomende DNA methylatie.

Het vermogen om genexpressie stil te leggen door gerichte DNA methylatie in celcultuur experimenten gaf aanleiding om de tijdsduur van de aangebrachte epigenetische markeringen ook in een kankermodel in muizen te bestuderen. De ZF-DNMT3A fusie werd hiertoe gekloneerd in het Tet- ON Dox induceerbare systeem en gevalideerd in celcultuur experimenten. Wederom had de expressie van ZF-DNMT3A een sterker permanent effect op *SOX2* down-regulatie, en op cel proliferatie, dan de expressie van ZF-SKD. Zodra Dox behandeling in de ZF-SKD cellen werd stopgezet, kwam *SOX2* expressie weer terug op zijn oorspronkelijke niveau. In de ZF-DNMT3A cellen resulteerde de geïnduceerde gen methylatie in stabiele genexpressie remming. Dit phenotype werd verder getest in een muismodel voor borstkanker. Inductie van expressie van ZF-DNMT3A leidde tot remming van tumorgroei *in vivo*, gedurende verscheidene weken na stopzetting van ZF-DNMT3A expressie (**hoofdstuk 6**). Bovendien, toonde immunofluorescentiekleuring van tumorsecties aan dat het SOX2 eiwit afnam na inductie van ZF-DNMT3A expressie, deze afname werd behouden tot 80 dagen na het stopzetten van Dox- behandeling. Deze down-regulatie

van *SOX2* expressie correleerde met afname van de proliferatiemarker Ki67, ook lang na stopzetting van ZF-DNMT3A expressie.

Concluderend laten we in dit proefschrift zien dat gen-gerichte DNA methylatie inderdaad leidt tot het remmen van genexpressie. Verder laten we zien dat DNA methylatie stabiel wordt overgedragen tijdens de mitose en de repressieve staat van het doelwit-gen in stand houdt.

ZUSAMMENFASSUNG

Bei der Kontrolle der Genexpression spielt die Methylierung der DNA an Promotoren eine wichtige Rolle und trägt damit zur korrekten Regulierung vieler biologischer Prozesse bei. Häufig sind die genomweiten Methylierungsmuster in Krebserkrankungen verändert, was mit dem Einsetzen und Fortschreiten boesartiger Tumorerkrankungen in Verbidnung gebracht wird.

ZIEL

Das Ziel meiner Dissertation galt dem Ausschalten von spezifischen Genen, die bei der Entstehung von Krebserkrankungen beteiligt sind. Um dies zu erreichen wurden Zinkfinger (ZF) Proteine verwendet, welche in der der Lage sind, je nach Design, exakte DNA-Abschnitte in der genomischen DNA zu erkennen und zu binden. Diese ZF Proteine wurden mit einer DNA Methyltransferase gekoppelt, welche DNA Methylierung induziert und somit die Genexpression unterdrückt.

Auf eine allegemeine Einleitung in Kapitel 1 folgt in Kapitel 2 wie ZF Proteine bausteinartig zusammengesetzt werden koennen. Dargestellt werden die exakten Schritte, die zur Herstellung von ZF Proteinen durchgeführt werden.

In Kapitel 3 wird beschrieben wie eine „ZF-Bibliothek" gefiltert wird, um ZF Proteine zu identifizieren, die in der Lage sind die endogene Expression von EpCAM zu regulieren. Die Expression von EpCAM ist sowohl bei der Verhinderung als auch bei der Entstehung von Krebserkraunken beteiligt, was von der jeweilgen Krebserkrankung abhängig ist. Um ZF Proteine zu identifizieren, die in der Lage sind die EpCAM Expression zu herbeizufuehren wurden die ZF Proteine der Bibliothek mit einem Protein (VP64) gekoppelt, das die Genexpression aktiviert. Im Gegensatz dazu, um die Genexpression von EpCAM auszuschalten wurden die ZF Proteine mit dem Inhibitor SKD verknüpft. Beide Bibliotheken wurden in drei Selektionsrunden auf ZF Proteine gefiltert, die die EpCAM Expression entweder aktivieren oder verhindern koennen. Bei dieser Selektion wurden jedoch keine ZF Proteine isoliert, die die EpCAM Expression verändern konnten. Um die Funktion bereits zuvor hergestellter ZF Proteine bei der Erkennung des endogenen EpCAM Promoters zu verbessern, wurde die Nukleotidsequenz, die für die Bindungsdomäne kodiert in einen retroviralen Expressionsvektor kloniert. Nach der retroviralen Infektion der modular hergestellten ZF Proteine wurde eine Aktivierung der endogenen EpCAM Expression erreicht, was später durch uns veröffentlicht wurde.

Um die Expression eines Onkogenes auszuschalten, wurde zusätzlich der Transkriptionsfaktor SOX2 ausgewählt. Wie enorm die Wirksamkeit unserer Desinger ZF Proteinen ist, um die Genexpression zu unterdrücken ist in Kapitel 4 dargestellt. Um zu beweisen, dass es möglich ist die endogene SOX2 expression zu verhindern, wurde zunächst ein nicht-permanter Inhibitor (SKD) verwendet. In zwei Zelllinen wurde die SOX2 expression durch unsere ZF-SKD Konstrukte verhindert und wird dargestellt, dass unsere künstlich hergestellten ZF Proteine ihre Zielsequenz im endogenen SOX2 Promoter binden. Darüberhinaus wird gezeigt, dass das Unterdrücken der SOX2 Expression dazu fuehrt, dass der Phenotyp von Krebszellen dramatisch verändert wird, dies zeigt sich bspw.

durch eine enorme Reduktion der Zellvermehrung und den Verlust der Fähigkeit Kolonien in Agar zu formen. Als nächstes wählten wir ein ZF Protein aus, um zu bestätigen, dass die phenotypischen Veränderungen gezeigt in Zellkulturexperimenten auch in einem Brustkrebsmodel im Tierversuch erhalten bleiben. Dazu wurde das ZF-SKD Protein in ein Doxycycline (Dox) induzierbares Expressionsystem kloniert. Dies erlaubte, dass sich die Expression des ZF Proteins durch Dox regulieren liess. Durch die Gabe (Fütterung) von Dox wird die Expression des ZF-SKD Konstrukts induziert und das Einstellen der Dox-Gabe führt zum Erlöschen der ZF-SKD Expression. Die Expression des ZF-SKD Konstrukts durch die Dox-Gabe, zeigt eine enorme Verringerung des Tumorwachstums im Mausmodel, welches mit einer dramatischen Reduktion der SOX2 Expression einhergeht und darüberhinaus die Expression des Proliferationsmarkers Ki67 ausschaltet.

Nachdem wir bestätigt hatten, dass unsere Designer ZF Proteine in der Lage sind ihre Zielsequenz zu erkennen und zu binden, war das nächste Ziel die Genexpression durch DNA-Methylierung permanent zu unterdrücken. Der katalytisch aktive Bestandteil der humanen DNA Methyltransferase 3A (DNMT3A) wurde durch Klonierung mit SOX2 und MASPIN spezifischen ZF Proteinen verknüpft. Kapitel 5 zeigt zum ersten Mal wie die gezielte DNA-Methylierung durch ein ZF-DNMT3A Fusionsprotein die Expression zweier Gene (das Onkogen SOX2 und das Tumorsuppressorgen MASPIN) permanent verhindert. Wie erwartet führt die Methylierung von SOX2 zum Ausschalten seiner Genexpression und zur Verringerung des onkogenen Potentials, während die Inhibierung der MASPIN Expression zu einem aggressiveren Phenotyp führte. Dies zeigt wie wichtig es ist, das die katalytische Aktivität des DNMT3A Enzyms durch spezifische DNA Erkennungsmodule zur Zielsequenz gesteuert werden muss. Wie erwartet führte die Inhibierung von SOX2 durch gezielte DNA-Methylierung zu einem stabileren Phenotyp im Vergleich zum Effekt der zuvor durch den transienten Repressor SKD erreicht wurde. Im Falle von MASPIN wurde die Genexpression für mehr als 50 Zellgenerationen durch das ZF-DNMT3A Fusionsprotein verhindert. Erwähnenswert ist hier zusätzlich, dass die Aufrechterhaltung der DNA-Methylierung während der Zellteilung durch den gezielten Knock-out von UHRF1 (ein zellulares Protein, das bei der Übertragung der DNA Methylierung beim Preplikationspozess notwendig ist) aufgehoben wurde. Somit haben wir bewiesen, dass die künstlich herbeigeführte DNA-Methylierung durch zelleigene Prozesse aufrechterhalten wird, genauso wie es bei der natürlichen DNA Methylierung bereits gezeigt wurde.

Da wir in der Lage waren die Genexpression in Zellkulturexperimenten durch gezielte DNA-Methylierung zu verhindern galt unser Interesse als nächstes zu überprüfen wie lange die künstlich herbeigeführte DNA-Methylierung im Tiermodel erhalten bleibt. Das ZF-DNMT3A Fusionsprotein wurde in das Tet-ON Dox-induzierbare Expressionssystem geklont und seine Funktion zunächst erneut in Zellkulturexperimenten charakterisiert. Nachdem Dox vom Zellkulturexperiment entfernt wurde zeigt sich, dass mit dem ZF-DNMT3A Protein eine weitaus stabilere Reduktion der SOX2 Expression, sowie eine Verminderung der Zellvermehrung herbeigeführt wurde als durch die Expression der ZF-SKD Konstrukte. Sobald der Repressor ZF-SKD nicht mehr exprimiert wurde, wurde die Expression von SOX2 mRNA und Protein wieder vollständig hergestellt, während die Methylierung des SOX2 Onkogenes durch das

ZF-DNMT3A Fusionsprotein, die Genexpression langfristig verhinderte. Dieser zelluläre Phenotyp wurde als nächstes in einem Tierversuch im Brustkrebsmodel überprüft, wobei die Expression unseres ZF-DNMT3A Konstrukts zur Inhibierung des Tumorwachstums *in vivo* führte. Zusätzlich zeigte die Analyse von Tumorschnitten durch Immunofloureszenzfärbung, dass die SOX2 Expression duch die Induktion des ZF-DNMT3A Proteins ausgeschaltet wird. Dieser Effekt wurde für mehr als 80 Tage aufrecht erhalten und ging mit einer Abnahme des Proliferationsmarkers Ki67 einher. Dies wurde selbst nach dem Entfernen der ZF-DNM3A Expression stabil aufrecht erhalten.

Zusammenfassend zeigen wir hier zum ersten Mal, dass die gezielte Methylierung zweier Gene mit ZF-DNMT3A Fusionsproteinen zur stabilen Verhinderung der Genexpression führt, welches während der Mitose durch zelleigene Mechanismen übertragen wird.

PUBLICATIONS AND BIOGRAPHY

LIST OF PUBLICATIONS

"Bidirectional modulation of endogenous EpCAM expression to unravel its function in ovarian cancer"
(van der Gun BT, Huisman C, Stolzenburg S, Kazemier HG, Ruiters MH, Blancafort P, Rots MG.; *Br J Cancer 2013*)

"Breaking through an epigenetic wall: re-activation of Oct4 by KRAB-containing designer Zinc Finger transcription factors"
(Juárez-Moreno K, Erices R, Beltran AS, Stolzenburg S, Cuello-Fredes M, Owen GI, Qian H, Blancafort P.; *Epigenetics 2013*)

"Reprogramming of Cancer Cells by Targeted DNA Methylation"
(Ashley G. Rivenbark, Sabine Stolzenburg, Xinni Yuan, Marianne G. Rots, Brian D. Strahl and Pilar Blancafort; *Epigenetics 2012*)

"Targeted silencing of the oncogenic Transcription Factor SOX2 in breast cancer"
(Sabine Stolzenburg, Marianne G. Rots, Adriana S. Beltran, Ashley G. Rivenbark, Xinni Yuan, Brian D. Strahl and Pilar Blancafort; *Nucleic Acids Research 2012)*
Significance: This article was highlighted by the journal for its innovation and scientific excellence and selected for the cover.

"Engineering Transcription Factors in breast cancer stem cells."
(Pilar Blancafort, Karla O Juarez, Sabine Stolzenburg, Adriana S Beltran; *InTech 2012*)

"*Modulation of Gene Expression Using Zinc Finger-Based Artificial Transcription Factors."*
(Stolzenburg et al, 2010; *Methods in Molecular Biology 2010*)

"Stable inherited oncogenic silencing in vivo by programmable and targeted de novo DNA methylation in breast cancer"
(Sabine Stolzenburg, Adriana S. Beltran, Ethan Ford, Theresa Swift-Scanlan, Ashley Rivenbark, Ryan Lister and Pilar Blancafort; *submitted*)

"Dynamics of genome-wide DNA methylation changes and its effect on histone modifications and gene expression"
(Ford E*, Stolzenburg S*, Grimmer M*, Farnham P, Blancafort P, Lister R.; *in preparation*)

"Genome-wide DNA binding specificity of engineered Zinc Finger Proteins"
(Grimmer M*, Stolzenburg S*, Ford E, Lister R, Farnham P, Blancafort P; *in preparation)*

BIOGRAPHIE

Sabine was born on September 1, 1980 in Erfurt, Germany. After obtaining her Masters degree at the University of Tuebingen in 2005, she started her PhD studies in the laboratory of Prof Marianne G. Rots at the University of Groningen in The Netherlands. Under the supervision of Marianne Rots, Sabine conducted research on targeting the Epithelial Cell Adhesion Molecule (*EpCAM*) using designer Zinc Finger (ZF) transcription factors until March 2009 at the University of Groningen. In order to indentify novel *EpCAM*-targeting ZF proteins, Sabine received a Boehringer Ingelheim travel grant to join the laboratory of Prof Pilar Blancafort at the University of North Carolina in Chapel Hill for six months. In January 2010 Sabine joined the lab of Pilar Blancafort permanently to conduct experiments targeting the transcription factor *SOX2,* which plays a major role in the oncogenesis of cancer, especially breast cancer. Sabine conducted experiments on the transient and permanent repression of *SOX2* using ZF proteins until September 2012 in Chapel Hill. This work resulted in the publication of two manuscripts on targeted gene regulation of *SOX2* using artificial transcription factors. Sabine was selected present her successful work in an oral presentation at the Era of Hope conference in 2011 by Department of Defense. In January 2013 Sabine transferred to the University of Western Australia in Perth and continued her work on targeted *SOX2* DNA methylation using ZF proteins. The results of the studies on *EpCAM* and *SOX2* are presented in this thesis.

ACKNOWLEDGEMENTS

The preparation of this thesis was an incredible journey filled with unexpected twists, turns and foremost travels. Little did I know when I left Germany that the years of my PhD thesis would take me to three different countries on three different continents. On this long journey I met so many great scientists, encouraging colleagues, outstanding students and made wonderful friends. This exciting journey and this thesis would have not been possible without the scientific and social support of many people, whom I want to thank on these last pages.

I would like to thank Marianne and Pilar for their scientific guidance throughout my PhD. Marianne: you said in the first meeting I had with you that you might get "pushy" at times. Yes, this is certainly true, but I am glad you did: I would not have given my first presentation at the Dutch Chromatin meeting only 4 months into my PhD if you did not believe that I can do it. The same counts for my research visit to North Carolina. I am very thankful of your support and trust, it made me grow as a scientist and a person. In Chapel Hill, I joined the lab of Pilar: thank you for taking me into your lab at UNC. I have learned a lot from you and not only from a scientific point of view. Your enthusiasm for science and positive thinking are both contagious and motivating.

When I started my PhD in Groningen Prof L.F.M.H. de Leij welcomed me to the Medical Biology Department with the following words: "Welcome Sabine, we have great hopes in you." At the time, this was quite honestly both a little intimidating and truly motivating. Thank you, Prof L.F.M.H. de Leij for taking part as my promotor and especially for taking the time to join my defense.

Thank you to my reading committee, Prof Rolf Sijmons, Prof Marc Timmer and Prof Albert Jeltsch, for taking the time to read and evaluate my thesis.

During my time at UNC I had the chance to work with Dr Theresa Swift-Scanlan and Dr Brian Strahl, a collaboration I truly enjoyed. With the same gratitude I want to thank Dr Ethan Ford and Prof Ryan Lister for your collaboration: you were of indispensable value to my scientific growth in my final year. Working with you was a great pleasure.

While I was working in three different institutions I have had a lot of colleagues who I enjoyed working with. I would like to thank Alice, Ieneke, Bram, Betty, Marieke, Marije, Anna, Inge, Roelof-Jan and Marloes for the time we worked together at UMCG. Fahime, we never worked together at UMCG, but who knew then that we would be colleagues again in 2014!

In North Carolina I joined the Blancafort lab at UNC, which was a great place to be at that time. We were perfectly placed right next to the always open door of Dr Lee Graves: thank you Lee for always having time and all the things you fixed for us. In the Blancafort lab, I met Sujey, a full time academic with two little kids. This was truly inspiring: I have lots of respect for the things you achieved, Sujey. Rob, Sujey, Xinni, Ashley and Bryan made it so easy for me to feel at home in the lab. Bryan, thank you for all the fun we had during normal, but especially late lab hours, harvesting viruses on the weekends and for so many great scientific discussions! You are an outstanding scientist and wonderful person. Thank

you to the entire Pharmacology Department, for the fun I had with you, your expertise and efficiency. Special notice here must go to Janeice, as she was the one who processed uncountable visa forms for me.

When I started to work at UWA, the Blancafort lab included only Pilar and me, but in no time we grew to a respectful number of people. Rabab, doing a PhD with two little kids, earns all my respect: You are a great scientist, hang in there: it will be ok! My experiences at UWA were mostly related to teaching and I will not forget my first students, Sufyaan, Rey, Sara and Jessie, not so much for your contribution to my thesis but for your trust in me and the things I learned teaching you. I hope you remember me as positively as I remember you! The whole Anatomy Department was filled with supportive, excellent staff: I had a great time working in this professional yet joyful environment. Thank you Cyril for sharing your adventures and experiences with me, you and Dijana made Europe feel a little bit closer. I also would like to thank Greg, Celeste and Zoe, my real 'uStrayan colleagues, for open doors, technical, scientific and social support: It was a great pleasure!

I would like to acknowledge all the great people I met during my PhD, who gave me life support and made me feel at home even when I wasn't:

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Dear Jihane, Fany and Mara, to have you around in the student house was a key contribution to me hanging in there, instead of going back to Germany already in the first month. I thank especially Fany and Jihane: the times I had with you in Groningen and the support I got from you during these first 2 years of my PhD, which felt like the hardest, were essential and will never be forgotten. Milena, thank you for getting exhausted with me at the climbing gym and sharing your experiences about moving labs during your PhD. Yes it is possible! Girls, I am so thankful you were there with me.

The core time of my PhD I spent in Chapel Hill, which was an extraordinary experience in all aspects of my life. Here I found wonderful friends, who became my family for four years, and it was really hard to leave behind the home I found in Chapel Hill: thank you Lorraine for all the things we shared! We went through a lot, good and bad experiences, and we survived them all. I wish you all the best. Maggie, from the way we met, I would have not thought we would become such great friends, I am glad we did anyways! Thank you for being such a wonderful source of energy and never ending support. Andi, Scottie, Ryan and Shub: it was my luck to be there with you, because Carrboro without you wouldn't have been the same! Many thanks also to my climbing people for all the fun on the wall, but especially for the trip to West Virginia for my birthday: it will never be forgotten! Altogether, I had such an epic time with all of you; thank you for being exactly who you are. Josh, thank you for caring, the great times on the porch and doing your best to be understanding when I was frustrated, annoying or just me. I know it wasn't easy at times! Haydee, you were a colleague at first, then my house mate and became one of my dearest friends! Meeting you made this great time in Chapel Hill even better than it was before. The friend I found in you and the support we found in each other is one of the greatest experiences of my life. Thank you so much!

The final year of my PhD I spent in Perth (I confess I had to look it up on the map after I agreed to this job). When I first came I was convinced I would not stay here any time longer

than was necessary, but in time I fell in love with this beautiful place, and a big influence on that were the people I met here. My climbing crowd: you were indispensable to me to go beyond my limits, a skill I took with me into the real world. Scott, your house is truly "a house of happiness", a harbour of stability. You made your home my home and I am deeply thankful for your friendship, our discussions, the many trips and just for letting me know who you are. Denise, it felt at first as if I got to know you way too late, now it feels like I have know you since a life time. Thank you for reaching out and being the rock during the tough times, your words of encouragement and the support, and thank you for your friendship since. Not to forget: this thesis wouldn't read as well without your help.

Meine Abenteuerlust ist tief verknuepft mit der moralische Unterstuetzung aus heimischen Landen und der Gewissheit "I can always go home". Die letzten sieben Jahre habt ihr mich nicht vergessen und aus der Ferne unterstuetzt. Anja, Dani, Sneska, Stephie: Danke fuer eure grenzenlose Freundschaft!

Aus tiefstem Herzen moechte ich meiner Familie danken, die immer an mich geglaubt hat. Keine Worte dieser Welt koennen ausdruecken welchen Beitrag ihr zum Gelingen dieser Dissertation beigetragen habt. Mami und Micha, danke dass ihr mir die Moeglichkeit geben habt meinen Weg zu gehen und meine Traume zu verwirklichen. Ihr wart in meinem Herzen immer dabei! Ihr habt mit gefiebert, mit gelitten, mit geeifert und mit gefreut, euch verdanke ich meinen Erfolg!

Sabine